

Tasks for a British science adviser

The British government's new chief scientific adviser would have faced a formidable task even if it had not been complicated by the abrupt transfer of the Office of Science and Technology to the Department of Trade and Industry.

SUBVERSIVE advice notwithstanding (see *Nature* 376, 281–282; 1995), all the signs are that Professor Robert May will indeed start work on 1 September as the chief scientific adviser to the British government. That happening will not, by itself, bring to a halt the grumbling there has been about the precipitate transfer of the Office of Science and Technology (OST) from the Cabinet Office to the Department of Trade and Industry (DTI). That will not happen until there is tangible evidence for the government's contention that the change of administrative location will make no difference. And the crucial test of that will be whether the new appointment will indeed enable the government to get a grip on the whole range of research supported by government departments, not simply that administered through OST itself. That is where the location of the office makes a difference. Officials from, say, the Ministry of Defence are less likely to show up at meetings convened on DTI notepaper than if the arrangements appear to have been made at or near the centre of government.

In fairness to all concerned, the government included, expectations of better coordination should not be exaggerated. For the past half-century, British governments have always had at least a token mechanism for coordinating policy on science and technology. First, there was a chief scientist enjoying post-war clout, then an Advisory Council on Scientific Policy (1952–60) with the freedom to publish its advice, briefly (1960–63) a powerful science minister and then (1964–70) a partly executive Council for Scientific Policy. Although at times (and especially after the election of the 1964 Labour Government) ministers have been deluged with scientific advice, in the whole of that long period (and since) there has been a general conviction that something has been wrong. For much of the time, defence research and development has been carried on as if it had no connection with the needs of civil industry, while public policy in general has been ill-informed about matters as different as the viability of military projects (who remembers the TSR2 aircraft?) and the speed of the communications revolution.

Rothschild

On paper, the then Lord Rothschild would have put all this to rights with his 1971 proposal that each government should think its way into the role of a customer for research, buying research services as it needed them from (mostly) public laboratories. That principle is seamlessly connected with the recent wave of decisions that this or that laboratory

or research organization should be hived off into private ownership. The more crucial part of the recipe, that each department should also have a chief scientist able to represent the needs of the ultimate customers (taxpayers) to the research providers, never appealed as strongly to ministers or to their civil servants. It was no part of the Rothschild recipe that there should be coordination of all government research; rather, he took the view that sufficiently able departmental chief scientists seeking the best value for money for their departments would be guided to the right policies by some version of Adam Smith's invisible hand.

Problems

Rothschild (sadly) is dead, and much of the research system for which he planned has disappeared. (Some of the old agricultural research stations are now directly responsible to the producers of particular crops, which goes further than even radical Rothschild would have done.) But problems remain, and may indeed have multiplied. Now that public support for defence research is channelled through the semi-autonomous Defence Research Agency, doubts about the wisdom of spending habits have been stilled only because they cannot as easily be voiced. Other departments, health and environment for example, are relatively big spenders on research, but in mostly predictable ways. At the very least, their programmes would benefit from the kinds of discussions to be heard every day over laboratory coffee. Luckily, May is just the kind of man to get such conversations going, provided that he can get his foot in the ministries' doors. Formal (and no doubt heavy-handed) coordination is no longer a need, but informal ways of keeping departmental research programmes alive (and their administrators alert) are a crying need.

That is half of a job description. The other, necessarily more complicated, concerns the way in which the British government regards the role of science in the modern world and in its own affairs. It is not alone among governments in being repeatedly surprised by new developments, from the emergence of molecular genetics as a potentially powerful ingredient of modern health-care to the impending disappearance of the distinction between television signals and broad-band digital communication (which calls into question existing regulatory regimes). The tendency of young people to defect from technical studies to softer (and ultimately better rewarded) options is similarly not just a British problem, but it is one that will have to be solved if



the decade ahead is not to ring with cries of "How do we keep up with China?". More, or better, public understanding of science is not a sufficient answer. It would be wrong to suppose that a science adviser, however able, would be able to solve these problems on his own; the post is, after all, an adviser's post, not one with ministerial power and responsibility. But May could help to change the way that ministers think.

There remains the business of talking and listening. Until the abrupt move of OST to the DTI, the British government had succeeded in stilling some of the most gruesome fears about the future of the British research enterprise by the willingness of OST's first minister, Mr William Waldegrave, and the head of OST, Professor William Stewart, to argue even unpopular cases in public. Openness, unsurprisingly, proved to be disarming. During the past two years, the result has been that the British research community has come to harbour a better appreciation of its own undisputed strengths. Why talking and listening should have this effect is not obvious, but is real enough; all policies are more persuasive when they are intelligible, which allows people to clear their minds for optimistic thoughts. If need be, May should take this task on his own shoulders, but science advisers everywhere should follow suit. □

Privatization in tears

Newly private British water companies are in trouble with their first summer drought, and deserve to be.

AMONG the endless difficulties provoked by spinning off public monopolies into private ownership (see *Nature* 376, 451-452; 1995), one has just come to light: what happens if a private monopoly cannot fulfil its obligations to its customers? The place is Britain, "the cradle of privatization", whose rainfall is as heavy as any other country in Western Europe, the commodity is water and the hapless victims of a long-standing summer drought are the customers of the private companies (whose share prices are publicly quoted) which assumed responsibility for water supply just a few years ago. They have hardly been out of the newspaper headlines this year, first for rewarding their senior officials more handsomely than seems necessary, now for requiring that their customers cannot feed water to the grass and flowers that they grow because of a shortage of supply, made worse by the leakage of up to a third of what is available from the distribution system. If the water supply industry were still nationalized, complaints at the restrictions of the past few weeks would have been muted; government edict carries clout. Properly, private companies are less able to act in such a cavalier way. What are they, poor things, to do?

A few simple remedies from elsewhere in the world would not come amiss. Why not sell water by the cubic metre and stop offering an *ad lib.* supply in return for an annual fee, which is the British practice? Unaccountably, the water companies are busily resisting that thought, saying how much the installation of water meters would cost, per-

haps £4 billion for the whole of Britain. But there is no way in which that reluctance can be sensible, either in economic or technical terms. Like all other commodity suppliers, the water companies suffer from peaks and troughs in the demand for their commodity, which means that their distribution networks must be more than minimally commodious. Their unique difficulty is that peak demand coincides with the times when the supply of surface water is at a minimum, which is why the companies imposing restrictions on water usage have been those whose supplies come from open reservoirs or rivers. There are probably as many people in Britain as there are elsewhere who would think twice about keeping a pet geranium alive if they had to pay *pro rata* for whatever it transpires each day.

That points to a wider issue. Water is not a scarce commodity in any temperate country, but the means for its collection, purification and distribution entail economic investment in reservoirs, pipelines and the like. At any particular time, the past volume of investment may be insufficient to allow the system to meet the present demand. (The water companies now say that they inherited many decades of inadequate investment, but why did they not arrange before they were privatized to borrow the money to make good the deficiencies?) But the needless or even feckless use of water, which may not matter when the supply is ample, is a waste of economic resources (and not of water) when demand and supply are out of balance. Economic signals that make that point, far from being anathema to the water industry, are essential for its well-being.

What can the poor companies do? One remedy is to invest more in the facilities of supply. That, too, will be expensive, of course, which is all the more reason for seeking to abate the horrors of peak demand by the use of water meters. (It is very curious that companies that have quickly learned to pay over-competitive salaries to their bosses should have been so slow to learn the rudiments of micro-economics.) But the equations could only come right in the long run. Indeed, the companies would find that they had a choice; either they could invest in facilities of their own, or they could buy pure water from elsewhere.

There are other ways of tackling the problem which have, by the technical standards of the water industry, futuristic components. In Britain, for example, why not ship water from one part of the country to another? This past summer, London has remained free of restrictions on the use of water because of the construction of a circular subterranean tunnel, at a depth of 300 metres, that serves both as a reservoir and a conduit for water. Why not extend that system a little further? Fears that the result would rob London of water would be legitimately offset by the knowledge that water from elsewhere would join the common pool. The water companies as they are would resist this notion also, saying that it would be uneconomic to invest such a large amount of money in guarding against a "1 in 50 years" drought. Wise water companies would inform themselves on global warming at this stage. To urge them on, the British government should make the water companies pay compensation to the customers they are depriving of water. □

Brussels enters debate over effects of nuclear tests on environment

Paris. France last week agreed to talks with the European Commission about the potential effects on the environment of its planned nuclear tests in the Pacific, and to let the commission visit its Mururoa Atoll test site to verify the control measures being taken.

The talks, due to be held today (Thursday 24 August), will focus on an extremely technical report on the precautions being taken by France to prevent radioactive emissions, which was submitted by France last month at the commission's request. The terms of the commission's mission to Mururoa will also be discussed at the meeting.

"This time, we considered we should take the initiative, and not wait until this information came to us after the tests," says Thierry Daman, a spokesman for the commission. The commission has argued that the 1957 Euratom treaty gives it the power to ensure that the safety measures accompanying tests are sufficient to safeguard public health.

Under the terms of the Euratom treaty, the commission can demand extra safety measures if it considers the tests to be "particularly dangerous". If, in addition, the tests risk affecting another member state — Britain has sovereignty over the Pitcairn Islands, 900 km from Mururoa — France would need authorization from the commission before proceeding.

But if the new commission, which was appointed earlier this year, is trying to flex its Euratom muscles, observers are sceptical that it would take the political risk of classifying the tests — which France considers are an area of national sovereignty — as "particularly dangerous".

Moreover, opposition to the French tests on the grounds that radiation may escape into the environment seems to be based more on speculation than on hard evidence, according to the various studies of the 41 atmospheric tests and 138 underground explosions that France has carried out in the Pacific.

A report submitted to a meeting last week of environment ministers from South Pacific countries, by a group of scientists from Australia and New Zealand, concluded, for example, that concentrations of radioisotopes around the atoll were low, and that potential releases through rupturing of the bedrock were unlikely.

But the report, which was largely drawn from three independent scientific studies carried out during the past decade, added that it was difficult to draw firm conclusions given that the studies had not been allowed complete access to the atoll.

The information office of the French armed forces (SIRPA), while hardly an

impartial observer, also claims that levels of artificial atmospheric radioactivity at Mururoa are lower than those of natural radioactivity in either nearby Tahiti or in France. SIRPA concedes that radioactive residues are present in the lagoon, but claims that these result from past atmospheric tests, and that their levels are similar to those found in the Baltic and North Seas.

"Everybody now agrees that radioactivity at the surface of the atoll is low", says Abraham Behart, a member of the Association of French Physicians for the Prevention of Nuclear War, who took part in studies of the health of Polynesian populations in 1990.

But Behart does not discount the hypothesis, proposed in the newspaper *Le Monde* by Pierre Vincent — a vulcanologist from the Observatoire de Physique du Globe at Clermont-Ferrand — that further tests could rupture the rock and release radionu-

Atom cloud has silver lining

San Francisco. Nuclear weapons laboratories in the United States believe funding is nearly certain for a series of major projects aimed at monitoring and verifying the country's weapons stockpile, following the announcement by President Bill Clinton that the United States will press for a ban on all nuclear tests, no matter how small, from next year (see *Nature* 376, 540; 1995).

Although Clinton's statement did not refer directly to either the \$123.8-million Dual-Axis Radiographic Hydrotest (DARHT) at the Los Alamos National Laboratory or the \$4.5-billion National Ignition Facility (NIF) at the Lawrence Livermore National Laboratory, both in California, it implied a promise of support for these projects, laboratory officials said.

According to observers, funding for a series of robust programmes at all three national weapons laboratories is virtually ensured by Clinton's emphasis that the United States would need safeguards to maintain a reliable stockpile under a comprehensive test ban — such as research to improve treaty monitoring, programmes in theoretical and exploratory nuclear technology, and maintaining the capacity to resume nuclear testing. "The general defence pie is shrinking, but some of the pie will grow," says Geer, a spokesman for Sandia National Laboratories, based in Albuquerque, New Mexico.

It is too soon to say which specific programmes will benefit. But a ban on hydronuclear tests, yielding less than four pounds of TNT equivalent, will mean greater reliance on computer simulation techniques, says

IMAGE
UNAMAGABLE
FOR A COPYRIGHT
FOREIGN
REASONS

Pacific peace: freer access to Mururoa would firm up conclusions on effects of tests.

clides from underground cavities. This argument is dismissed by Alain Barthoux, director of nuclear tests at the French Atomic Energy Commission, who asserts that while fissures have been observed in the surface coral, the underlying basalt "remains stable".

Catherine Tastemain

Kathy DeLucas, a spokeswoman for Los Alamos, and an increased justification for the \$794 million Accelerated Strategic Computing Initiative. This ambitious five-year programme is aimed at linking the three laboratories with hardware manufacturers to develop extremely powerful three-dimensional modelling techniques.

DeLucas also says that Clinton's decision will provide "stronger arguments" for the continued funding of the DARHT facility at Los Alamos, which has been vigorously opposed by environmentalists. This high-explosives firing site, which is equipped with two state-of-the-art flash X-ray machines as a new tool for evaluating nuclear weapons, is due to seek a second round of funding in 1997.

Similarly, Lawrence Livermore says the new situation improves the prospects for its planned National Ignition Facility, which will study fusion using enormous lasers that can unleash up to 500 terawatts of energy onto a pellet of deuterium and tritium, compressing the pellet, heating it to more than a million degrees and triggering sustained thermonuclear fusion.

Such programmes are needed to retain competence in nuclear weapons, argue the laboratories. "The foundation of our capability has always been the competent experts on whom we rely", says C. Paul Robinson, president of Sandia National Laboratories. "It is crucial that we maintain the ability to keep these experts immersed in the relevant technologies that are so unique to nuclear weapons systems."

Sally Lehrman

Smoke signals snuffed out in San Francisco

San Francisco. A Congressional Committee has recommended the axing of funding for a research project which has revealed links between legislators and tobacco interests, and brought damaging internal tobacco industry documents to the attention of the public.

The three-year, \$600,000 grant for work being carried out by Stanton A. Glantz, professor of medicine at the University of California at San Francisco, was singled out in a report to the National Cancer Institute (NCI) by the House Appropriations Committee with responsibility for Congress' \$12 billion allocation to the agency.

Representative John Edward Porter (Republican, Illinois) has told NCI to stop the grant to Glantz. The research should not have been funded by NCI, or any of the National Institutes of Health, he said, on the grounds that it involved political and social science, not medical or clinical research.

Glantz has published nearly a dozen reports in peer-reviewed journals in the past

grant proposal, but merely felt "strongly" that such research fell outside NCI's mandate. Dave Kohn, a spokesman for Porter, says Porter hoped the National Institutes of Health would ask the Department of Health and Human Services to continue funding for Glantz's work through its discretionary funds.

But Glantz says that the agency had solicited work on the public policy aspects of tobacco control, and that his grant was just one of several in this area. "Just as you need to understand a vector of a disease to control it, the tobacco industry is the vector for lung cancer and heart disease," Glantz says. If Porter is successful, he adds, "that's going to undermine public confidence in the scientific community and the whole process".

Dr Tom Novotny, an epidemiologist and adjunct professor at the UC-Berkeley School of Public Health, says Glantz's work is essential to understanding the root cause of lung cancer and other diseases caused by smoking. The decision is an insult to academic freedom, he says. "It's a bit frightening because it injects politics into the determination of the way scientific research is carried out."

NCI, which is not legally required to obey the directive, has not yet taken any action. Donald Shopland, coordinator of the institute's smoking and tobacco control programme, said there may be legal restrictions on withdrawing funding from a current grant. Shopland added that Glantz, who is seeking renewal of his grant next year, probably would not receive further NCI funding for such work.

Kohn said Porter's committee took up the issue following an inquiry by the *Washington Times* of allegations by a group of taxpayers in Kentucky — a state where the tobacco industry is important economically — that NCI was using taxpayer money for political ends.

Porter investigated and reluctantly concluded that the group had a case. According to Kohn, Porter feared that opponents of the institute would use the issue to damage the agency. Porter does not represent a state with heavy tobacco interests, and has voted against tobacco industry subsidies, and in favour of a permanent ban on smoking on domestic airline flights. NCI officials also said the legislator had an excellent record for supporting NIH. **Sally Lehrman**



year alone, on the basis of work paid for by the NCI grant. The *Journal of the American Medical Association* recently devoted an entire issue to an analysis by Glantz's group of internal documents from Brown & Williamson Tobacco Co., which asserted that the company knew of the cancer risks associated with tobacco much earlier than the scientific community, and tried to cover up its findings.

A study published last year also claimed that state legislators who receive money from the tobacco industry are 42 times more likely to vote in favour of the industry than those who do not. Glantz is now probing the political activities of the tobacco industry in six states.

Porter said that the committee was not passing judgement on the quality of Glantz's

\$1 billion R&D spender emerges

Oxford, UK. Sweden's Pharmacia and Upjohn of the United States last week agreed to merge to create one of the world's ten largest pharmaceutical groups.

The merger continues a trend of consolidation among the world's pharmaceutical companies, prompted by the need to cut costs and improve efficiency because of pressure from increasingly budget-conscious governments.

It is also predicted that only the biggest companies will be able to afford the soaring costs of the research and development needed to produce blockbuster drugs that command high prices.

Had the new company, which is called Pharmacia & Upjohn, existed last year, it would have had 1994 sales of nearly \$7 billion, 34,500 employees and an R&D budget of more than \$1 billion. The company anticipates cutting its workforce by 4,000, but declines to say where cuts would be made. Sales and marketing positions, where most duplication occurs, are likely to be the main target, but R&D jobs may not be immune.

Pharmacia & Upjohn is likely to turn to its researchers as a source of new growth. "Uniting the respective R&D strengths of Pharmacia and Upjohn in molecular biology, chemistry and pharmacology and other key disciplines will create a powerful engine for innovation and new product

development," explains John L. Zabriskie, who is currently chairman and chief executive officer of Upjohn, and who has been proposed as president and chief executive of the new company.

Indeed, executives at the new company believe the \$1 billion it will be spending on R&D — a sum now considered as the minimum for world-class players — will bind the complementary strengths of both companies' current R&D activities and further accelerate revenue growth in the new company's major therapeutic markets.

Pharmacia & Upjohn has a strong pipeline of new products and indications. By 1998, the company expects to launch 18 new chemical entities and 12 new major indications and line extensions. The new company also has 24 products in early-phase clinical testing, including promising cancer and central nervous system drugs, and has one of the world's strongest biotechnology capabilities with more than 1,000 scientists experienced in recombinant and monoclonal technologies. This should enhance the company's oncology and inflammation activities.

But while it says it will be a research intensive company, the management clearly believe that cost reductions — of more than \$500 million a year — will make a substantial contribution to improved profitability. **Mike Ward**

Energy department X-rays skeletons in Cold War closet

Washington. The US Department of Energy (DoE) last week released final estimates of the number of radiation experiments carried out on humans by the department and its predecessors, including the Atomic Energy Commission, after the Second World War. The figures are the outcome of a controversial 18-month investigation involving the study of around 3.2 million cubic feet of archives from the Cold War era.

The DoE search is part of a government-wide investigation ordered by President Bill Clinton that also involves the Department of Health and Human Services, the Pentagon, the Central Intelligence Agency and other agencies. Clinton has also set up a panel of outside scientists, ethicists and physicians to advise the interagency committee that is coordinating the investigation.

The multimillion dollar study was initiated after media reports on such experiments triggered a public outcry. The study has been criticized by officials from other agencies, who fear it has unnecessarily alarmed the public and wasted valuable government resources (see *Nature* 368, 781; 1994).

The DoE estimates that 435 such experiments were carried out on around 16,000 subjects between 1946 and 1974. Ninety-five per cent of the studies involved either biomedical research or testing of diagnostic techniques; only around 20 experiments involved study of the biological effects of radiation. The department has made the list of experiments, including 250,000 pages of scanned historical documents, available on

the Internet at <http://www.ohre.doe.gov>.

The total number of experiments carried out by all government agencies over the same period is already estimated at more than 4,000, says Steve Klaidman, a spokesman for the panel advising the investigation. Very few could have caused "demonstrable harm" to subjects, but "very substantial numbers" raise the sort of ethical questions that have brought the issue to public and congressional attention. Subjects were sometimes not informed of the nature of experiments, while others involved prisoners, mentally retarded children and pregnant women. The advisory panel will decide whether the experiments violated the practices of informed consent at the time. While the issue of state compensations remains under discussion, legal proceedings have already been filed by research subjects.

But Geoffrey See, a spokesman for the Washington-based Task Force on Radiation and Human Rights, a victims' rights organization, points out that similar cases, such as those brought by nuclear bomb test veterans, have been unsuccessful in the past. The law tends to protect the government in areas of national policy, he says.

But in its draft report, the advisory panel recommends the government deliver "a personal, individualized apology and provide financial compensation" in very few cases, for example where subjects "both suffered physical injury and were deceived as to the benefit of the procedure". The final report will be published next month. **Tony Reichhardt**

Nuclear bomb site may go solar

Boston. The US nuclear weapons testing site in the state of Nevada is one of five candidates being considered for a "Solar Enterprise Zone" by the Corporation for Solar Technology and Renewable Resources (CSTRR), which is based in Las Vegas.

CSTRR received US\$3 million seed money from the Department of Energy (DoE) this summer to build a 100 MW solar power station in the state. Creation of big plants, the government hopes, may help make electricity from solar plants competitive with that from other sources.

The sunny DoE test site is a leading candidate for the plant. But CSTRR — which says it could generate 10,000 MW using just 7 per cent of the vast test site — will not make its final choice until January.

Underground explosions in Nevada ceased in 1992, when President George Bush introduced a moratorium on testing. The site is now on standby, ready to resume testing at two years' notice. Meanwhile, a

defence authorization bill has called for studies of alternative uses of the facility.

At the peak of nuclear testing, the site had a workforce of 10,000, but this has fallen by about half. The DoE grant, which will cover CSTRR's operating costs for two years, came from funds aimed at re-employing staff made redundant at the site.

The \$3-million grant is tiny, both in terms of total US spending on renewable energy and of the sums allocated to reconvert staff employed in the weapons programme. It is also small compared with the total cost of the solar plant, which has been estimated at about \$150 million. Rose McKinney-James, CSTRR's interim president is, however, confident that it can raise the needed capital. "Our non-profit tax status will help us with bond financing," says McKinney-James, who, as head of Nevada's Business and Industry Department, has close ties with corporate interests throughout the state. Steve Nadis

Low-flying biologists set to become endangered species

Washington. The US National Biological Service (NBS), which is deeply unpopular with many conservative Republicans in the US Congress, has won a stay of execution in the Senate.

But NBS faces further curbs on its activities from legislators concerned that government-sponsored biological research will cause new restrictions on property owners.

NBS received a budget of \$146 million for next year from the Senate committee that handles appropriations for its parent agency, the Department of the Interior. Although this is less than the \$173 million it requested, it is much better than the \$112 million granted in the House of Representatives, which also tried to abolish NBS by transferring its operations to the US Geological Survey.

The Senate-approved budget, say NBS officials, will be enough to maintain the agency's field centres and major programmes.

But the Senate also passed an eleventh-hour amendment by Senator Kay Bailey Hutchison (Republican, Texas) that will require NBS scientists to obtain written permission for aerial surveys of private property.

Property owners in Texas had complained about NBS-funded planes flying over their land, explained Hutchison. The House has already obliged NBS researchers to obtain written permission from landowners before working on private property, while volunteers must be properly trained and have their work verified.

If the amendment becomes law, it will end "important NBS and joint State, Federal and private partnership research efforts", NBS said in a statement.

Aerial surveys are used, for example, to provide the estimates of waterfowl populations for setting federal hunting limits. NBS says that it hopes the bill's wording can be changed to exempt most forms of aerial survey.

Still in doubt, however, is whether aerial surveys would be allowed to be used to continue verifying remote-sensing data gathered by satellite within Gap Analysis, an NBS-led programme to map biological resources across the US.

Hampering such research may not perturb Hutchison. She has also sponsored legislation calling for a moratorium on the listing of new species under the Endangered Species Act, and is concerned that aerial data gathered without the consent of property-owners might eventually result in their land being listed as a protected habitat. **Tony Reichhardt**

European Union research finds Scandinavian model

Munich. Jorma Routti, president of the Finnish innovation agency, SITRA, was this month formally appointed director general of the European Commission's research directorate, although he will not move from Helsinki to Brussels until the autumn.

Routti's first task will be to oversee six new technology development programmes — in vaccines for viral diseases, educational software and multimedia, integrated transport systems and the next generation of cars, trains and aircraft. These are being prepared by joint task forces, which Edith Cresson, the research commissioner, created earlier this year with the industry and transport directorates (see *Nature* 374, 206; 1995).

The task forces have been criticized for giving industry a greater role in deciding research policy — the commission has a poor track record in picking industrial winners. Some commission officials also believe the programmes will be too complex to administer. Many are also concerned that Cresson may divert the ECU700 million (US\$924 million) held in reserve for the next Framework programme from science programmes into pilot programmes suggested by the task forces. According to one commission official, the money will probably be divided between the pilot programmes, international cooperation — particularly with eastern Europe — and some oversubscribed programmes within the fourth Framework.

Nonetheless, Routti, who claims to be a staunch defender of basic research, enthusiastically supports the task forces, which he says have different goals from those of the Framework research programmes. Entering new high-technology markets and competing internationally require both flexible structures and combining the resources of the

research commission with those of other commissions, he says.

One key issue is whether the joint development programmes will be funded within the fifth Framework programme, a draft of which must be submitted to the European Parliament in 1997. Routti says he has not yet had time to consider the issue, but that basic research must be defended within



Routti: "flexible research structures needed".

Framework programmes. "Science and research should not be harnessed to pull the wagon of industry," he says. "Their economic potential needs to be safeguarded."

Routti's belief in a strong basic research base combined with efficient technology transfer is based on his experience in Finland. Forestry-related industries accounted for most Finnish exports up until the 1960s, but high-technology products now make up one-fifth. The continued strength of the forestry and related industries in world markets, he says, is a result of investment in research over the last two decades.

Routti, who is 56, has a background in physics and technology management. He became president of SITRA in 1986 and was responsible for reorganizing the agency, which promotes technology transfer by co-financing a system of cooperation networks between universities, venture capitalists and industry. SITRA has been instrumental in bringing about the acknowledged success of Finland in high technology. **Alison Abbott**

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Routti's belief in a strong basic research base combined with efficient

Japanese take first steps towards gene therapy trials

Tokyo. Japan's first application of gene therapy emerged from a long process of approval this month when researchers at Hokkaido University Hospital reintroduced lymph cells to a four-year old-boy suffering from adenosine deaminase deficiency, a rare enzyme deficiency that depresses the immune system.

Using a technique developed by the US National Institutes of Health in 1990, the lymph cells, extracted a week earlier from the boy, had been treated with a vector to introduce genes producing the deficient enzyme. The treatment will need to be repeated at monthly intervals, and the outcome will not be known for several months. Coming after a lengthy approval, this first gene therapy trial has obtained wide coverage in the Japanese media.

David Swinbanks

Murder charges brought in German HIV blood products case

Munich. Two officials of Haemoplas, a German blood products company, have been charged with murder for allegedly selling blood products not tested for HIV.

If convicted, Frank Giesbert, the director of the company — which is based in Osterode in the state (*Land*) of Niedersachsen — and Günter Eckert, the head of its blood product testing laboratory, face life imprisonment.

Laws testing blood products have been tightened following allegations last year that the Koblenz-based company UB Plasma pooled blood donations before testing for HIV, and visually assessed the colorimetric results. Three customers were infected with HIV, and one has died of AIDS. Five officials were charged with grievous bodily harm, which carries a maximum five-year prison sentence.

Giesbert and Eckert have been charged with murder on the grounds that they had been motivated by greed. They are alleged to have conspired in the early 1980s to economize by not testing blood samples, and to have persisted after testing was made compulsory in 1985.

Fourteen people became infected with HIV through Haemoplas products between 1986 and 1987, and three have since died.

Both men have also been charged with 5,837 counts of attempted murder — corresponding to the number of batches distributed — infringing legislation on the control of pharmaceutical products, and fraud.

Alison Abbott

EU rejects reshuffling renewables

Munich. The European Commission has dismissed as "absolutely untrue" allegations by the European Parliament's Green group that it diverted funds destined for renewable energy into other non-nuclear energy research.

Renewable energy should receive 62 per cent of the ECU967-million (US\$1.3-billion) funds from the five-year Joule non-nuclear energy programme. But of the ECU191 million distributed in the first round of grants in July, it received just 40 per cent.

One reason why suspicions of manipulation of funds have been aroused, the commission concedes, is that some applicants for renewable research projects had been told informally but incorrectly that their applica-

tions would be successful.

The commission claims the 62 per cent target was not met in the first funding round because "there were simply not enough good renewable energy projects. One hundred and seventy-six projects were chosen from 936 applications, on the basis of merit," says one official, who claims that the balance will be adjusted in subsequent rounds.

Proposals for renewable energy research tend to come from small companies and research groups that lack experience in preparing applications, he argues, adding that the commission now intends to coach such applicants, in a bid eventually to meet the 62 per cent target. **Alison Abbott**

"Virtual" science centre on road to reality

Potsdam. The uncertainty hanging over plans to build a science centre at Golm near Potsdam, capital of the east German *Land* of Brandenburg, was alleviated earlier this month following the local government's approval of funding for construction of the first building.

The move comes only a few weeks after Hans Zacher, president of the Max Planck Society (MPS), claimed that the Brandenburg ministry of science and culture was reneging on its promise to establish the University of Potsdam's faculty of science at Golm. MPS had decided to build three institutes — for colloid and surface research, gravitational physics and molecular plant physiology — at the site on the basis of the ministry's promise.

Before reunification, Brandenburg had

A site at Golm, which was the headquarters of military espionage during the 1930s, and later a training school for the Stasi, was chosen as the location of the science centre. The science centre was originally scheduled to begin operating next year, but until now it has seemed unlikely that building work would even begin by that date.

Speaking at a meeting in June, Zacher warned Brandenburg that failure to honour its commitments could cause MPS to move its institutes elsewhere.

"New unforeseen difficulties for the state," he said, "also put our plans in question."

The university believes that Zacher's strong words shamed the Brandenburg

government into action, after months of vacillation. But Heinz-Ulrich Schmidt, head of science at the ministry of science and culture, argues that the decision, which he says came after a long but routine bureaucratic process, was in any case expected this month. "Golm is a very high priority for us," he adds.

Schmidt says that the first building of the science faculty could be operating by 1997. The university, which complains that the science faculty is at present in unsuitable and overcrowded offices in the New Palace, says that a date near the end of the decade is more realistic.

The MPS now says that either starting date would be acceptable.

Alison Abbott



Palatial accommodation: plans to build Potsdam University were approved in 1991.

no universities. Recommendations to build three, along with five Fachhochschulen (technical colleges), were later made by Germany's science council, the Wissenschaftsrat, which assumed responsibility for assessing the science and university landscape in the former East Germany.

Plans to build Potsdam University were subsequently approved in 1991. The historical New Palace, built for Frederick the Second ("Old Fritz"), king of Prussia during the Napoleonic era, was proposed as the site of the main campus; Babelsberg, an eastern suburb of Potsdam which was the headquarters of the German Red Cross during the Third Reich and later the site of the Jurisprudence Academy, the hub of the East German ideology machine, was chosen for the law faculties and some arts departments.

South Africa's ivory towers turn towards townships

Cape Town. The goals of publicly funded research in South Africa's universities, technikons and museums will be reorganized over the coming months to meet the needs of the Reconstruction and Development Programme (RDP), a scheme which enjoys cross-party support in the government of national unity and which is intended to improve basic living conditions and promote economic growth.

The Foundation for Research Development (FRD) now spends R70 million (US\$19 million) annually, with around 60 per cent available to any area of research.

But FRD will end its existing funding schemes at the end of the year. Meanwhile, researchers have been asked to submit proposals within four new strategic themes: "Competitive industry", "Improved quality of life", "Sustainable environment" and "Effective science, engineering and technology education and awareness".

To take part in the new programmes, researchers must submit by the end of next month their proposals for grants of up to five years, although many have complained that the deadline is too short.

More general proposals will also qualify, provided they are aimed at "building and enhancing research capacity", while money will also be available for studentships, fellowships and equipment and for developing a research culture in historically disadvantaged institutions.

FRD says that all proposals must be internationally competitive, involve collaboration with industry and include

measures that favour blacks and women. Specific budgets will be attached to each programme only after the volume, quality and costs of the submissions has been assessed, according to Gerhard von Gruenewaldt, vice-president of the FRD.

FRD's plans have been applauded by many scientists as a careful response to the challenge to the country's scientists to "come up with suggestions on how they can contribute to the development of South Africa", made earlier this year by Bernie Fanaroff, deputy director-general of RDP (see *Nature* 374, 664; 1995).

But Roy Siegfried, director of the Fitzpatrick Institute of Ornithology at the University of Cape Town, expresses the concerns of others when he describes FRD's plans as an "over-elaborate attempt to partition research into themes which have areas of considerable overlap".

Siegfried argues that FRD should have delayed its reorganization until the release of a government white paper on science and technology policy that Ben Ngubane, the minister for arts, culture, science and technology, has promised will follow the soon-to-be-finished audit of the activities of the research councils.

The specific programmes and their contents were chosen on the basis of a broad consultation with the scientific community, says von Gruenewaldt.

But the increased emphasis on applied research in FRD's plans has also caused concern that this may be funded at the expense of basic research unless extra money is forthcoming from the government.

Michael Cherry

Australian universities

SIR — Mark Lawson (*Nature* 375, 623; 1995) reports that “four of Australia’s universities account for nearly half of the research effort in the national higher education sector, according to figures released [by the Australian Vice-Chancellors’ Committee] last week”.

In the text associated with the release of the figures, it was made clear that the Institute of Advanced Studies at the Australian National University (and the Australian Defence Force Academy at the University of New South Wales) were excluded from the data collection because they do not receive research quantum funding from the Department of Employment, Education and Training.

F. S. Hambly

(Executive Director)

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SIR — If one does not manipulate the numbers, the research output, impact and cost effectiveness in Australian universities continue to be dominated by the Institute of Advanced Studies (IAS) and the faculties of the Australian National University (ANU), not by the “sandstone club”. For example, data in *The Australian* (8 March 1995) show that ANU is responsible for 24 per cent of Australian publications in science that found their way into the top 1 per cent of world citations in all fields in 1988–92. The University of Melbourne accounted for 16 per cent, of Sydney for 12 per cent, of New South Wales, for 6 per cent and of Queensland for 2 per cent. This dominance of research impact by the ANU was achieved through the expenditure of only 12 per cent of all funds available for research for Australian universities over the period (Science and Technology Budget Statement 1995–96).

Recent reviews of the research schools in IAS, by some 70 distinguished researchers, have all commented on the extraordinary impact and effectiveness of the IAS block-funding model for university research. In the case of my own school, reviewers observed “the success of RSBS in this respect provides a model to be followed internationally”. On the eve of its fiftieth anniversary (1946–96) the ANU has proved to be one of the most successful experiments in the support of basic research in the natural and social sciences in universities ever undertaken in Australia or anywhere else.

One wonders why the evidence is so frequently ignored or misrepresented by those responsible for the support of science through the public purse, especially those who should be taking pride in these achievements. There is a clear need to sustain the successful model and repro-

duce it, so far as is possible, in the rest of the diverse university system. Instead, the prevailing wisdom seeks further constraints through outdated concepts of centralized funding, priority management and contestability (Industry Commission Enquiry into R&D 1995). Of course, there is no rush to review the achievements of these alternative models, but evidence of their profligate waste, failed priorities and lost opportunities litters the science policy landscape. Like other litterbugs, the offenders simply move on to offend again in another place, and are rarely called to account. It’s time to question accountability where it really matters, and let basic research find again the paths to our knowledge-based future.

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Striking contrast

SIR — The Commentaries of Benno Müller-Hill on “The shadow of genetic justice” (*Nature* 362, 491; 1993) and Robert J. Pokorski on “Genetic information and life assurance” (*Nature* 376, 13; 1995) are in striking contrast.

Pokorski seems worried about the profits of the insurance business; Müller-Hill is concerned for the welfare of the insured and the uninsurable. But happily Pokorski indicates, perhaps inadvertently, the solution to Müller-Hill’s problem: it is necessary only that private life insurance (based on ‘equity’) should be abolished, and public life insurance (based on ‘equality’) extended to all.

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Useful Usenet

SIR — I was appalled by your statement that “most Usenet postings are of no interest” (*Nature* 376, 200; 1995). There are thousands of serious and useful news groups, including many active scientific groups. Condemning the whole Usenet because it contains frivolous and even offensive sections is the height of indiscriminate. Groups such as alt.lenora.bobbit.chop.chop.chop are the price of free speech.

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Polio eradication

SIR — A recent leading article¹ suggests that “the number of new cases of polio this year is unlikely to exceed some four-digit number”. This refers to reported cases: the World Health Organisation (WHO) estimates that reporting efficiency is less than 10 per cent and that 110,000 cases of polio occurred in 1993 (ref. 2). Great progress has been made, and national Sabin days in March, April and May this year will have distributed two doses of oral polio vaccine (OPV) to nearly 70 million children under the age of 5 in the Middle East as far as Pakistan, in Trans-Caucasia and in the Central Asian Republics³. But most cases occur in the Indian subcontinent and we know very little of what occurs there. Why did about 90 per cent of cases not attend hospitals? There has been a 90 per cent drop in the number of cases attending surveillance hospitals, representing an impressive drive to give OPV. We do not know if there has been a similar drop in the number of those who would not have gone to a hospital.

When the Salk vaccine was first manufactured on a large scale in 1955, there were serious problems that led to 193 vaccine associated cases (VAC) with paralysis of which ten died⁴, a very small number compared with those protected that year from paralysis by wild viruses. The Salk vaccine (IPV) has since proved to be safe and effective. The Sabin OPV causes about one VAC per million children immunized: whether this is due to reversion of the virus, genetic susceptibility, other factors such as injections or a combination of all of these is not known. WHO now does not include VAC in its numbers with polio, so that the zero cases of poliomyelitis in the Americas since 1992 excludes VAC. In the United States the no-fault National Childhood Vaccine Injury Act of 1986 (NCVIA; PL 99-660) removes liability from the manufacturer although there are still some causes disputed by the government.

There are four critical indicators of surveillance for polio, yet by 26 November 1994, Haiti had met none, and Uruguay, the Dominican Republic and Costa Rica each met only one⁵. How soon India and other countries will be able to meet these criteria is uncertain.

Eradication of polio may be possible, but we know little of the epidemiology of polio in developing countries.

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1. *Nature* 374, 663 (1995).

2. Progress towards the Eradication of Poliomyelitis, Status Report March 1994, WHO/GPV/POLIO/94.1 (WHO, Geneva).

3. *Lancet* 345, 786 (1995).

4. Nathanson, N, Langmuir A.D. *Am. J. Hyg.* 78, 16–81 (1963).

5. EPI Newsletter 16 (6), 6, (1994).

Imprisoning the beams of the Sun

Science remains low on Pakistan's list of political priorities. Money and management both seem to be in short supply — except for nuclear energy which has benefited at the expense of other research fields.

PAKISTAN is approaching an important juncture in its brief but tumultuous history. In August 1997, 50 years will have passed since Lord Mountbatten presided, as viceroy, over India's partition into two independent countries. The occasion will be celebrated in considerable style. But it has also plunged Pakistan into a state of collective soul-searching about its turbulent past and uncertain future.

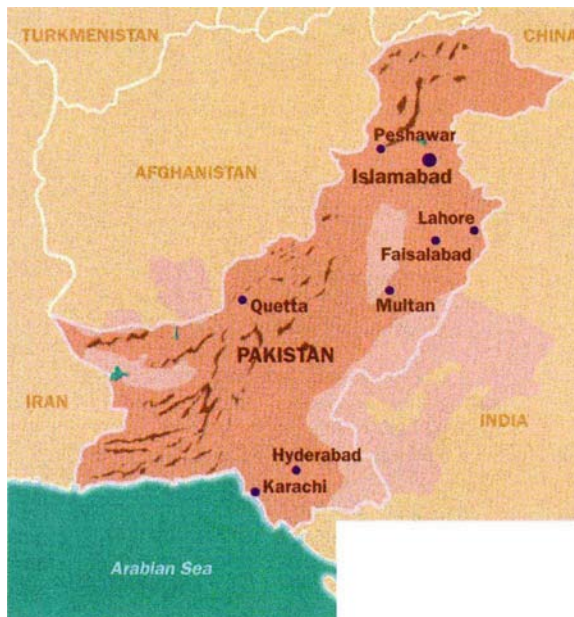
The government is expected to emphasize as Pakistan's biggest achievements its strategic importance, regional stability and open economy. But a former cabinet minister admitted recently that no amount of gloss can hide Pakistan's two most spectacular failures: education and science.

Adult literacy is only 30 per cent, post-primary education is not compulsory and only three per cent of 17–24-year-olds enter university. Eleven universities existed for a population of 65 million in 1971, when East Pakistan splintered off to form Bangladesh. Today there are 22 universities, but the population of what used to be West Pakistan has soared to 130 million and continues to rise by 3 per cent a year. It is ironic that a part of the world which, centuries ago, played an influential role in the evolution of science, today is a shadow of its historic past.

Pakistan's science base continues to teeter on the shaky foundations it inherited nearly 50 years ago. The country produces on average fewer than two science PhDs per university each year. This figure is in fact an improvement on the past. Pak-

istan produced a grand total of 128 PhDs between 1947 and 1986.

Half of last year's doctorates came from just one of Pakistan's designated 'centres of excellence', the Husein



Ebrahim Jamal (HEJ) Research Institute of Chemistry at the University of Karachi. Atta-ur-Rahman, the institute's director, says he does not know whether to celebrate or commiserate. "This may be a matter of satisfaction for us", says Rahman, author of *Science and Technology in Pakistan, a Matter of National Survival*. "But it is of grave concern to the country. If the present state of affairs continues, we will have very little to celebrate in 1997."

The Ministry of Science and Technology has lacked a minister for more than a year. S. M. Quraishi, a civil servant close to retirement, holds the fort when he is not doing his other job as chairman of the Pakistan Medical Research Council. Neither Prime Minister Benazir Bhutto nor President Farooq Ahmad Leghari — both alumni of the University of Oxford — has a science adviser. The prime minister's adviser on social issues, Shahnaz Wazir Ali, acts as a government spokeswoman on science, although she freely acknowledges her ignorance on scientific matters.

Leghari has publicly acknowledged the

"dismal state of science" in Pakistan. Inaugurating a recent conference on science policy in the Muslim world, the president asked that his audience of scientists should "honestly assess our predicament and find a way out of this impasse". Atta-ur-Rahman says that the president is aware of the problems from his meetings with scientists — the critical lack of funding, deplorable mismanagement and an absence of meaningful goals. But politicians, he says, "have no idea what science can do for them. An investment in science is often considered a waste of national resources."

Basit Hasan, a former research chemist who spent nearly a decade in the scientific civil service and went on to write *The Broken Ladder*, a book on the history of Pakistani science policy, cites the example of the Pakistan Council of Scientific and Industrial Research (PCSIR), the largest of the research councils that fall under the Ministry of Science's remit.

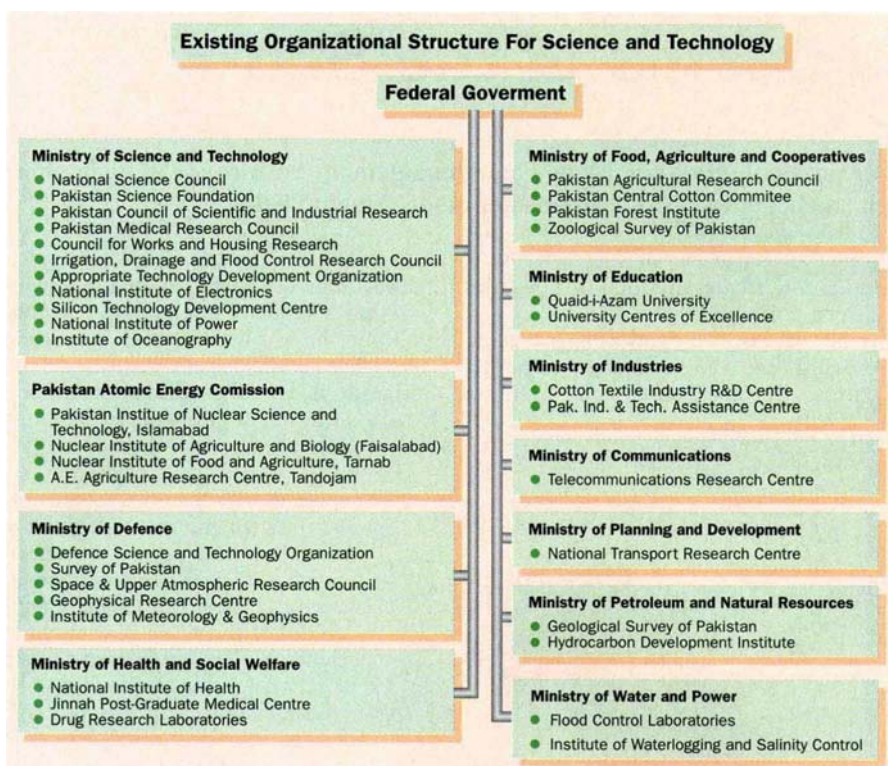
The PCSIR's nine research laboratories, he says, share an annual budget of roughly Rs187 million (US\$6 million). Seventy per cent of this is consumed by salaries and allowances. Half of the rest is spent on electricity, gas, telephone and transport bills. Sources at the Ministry of Science confirmed that the PCSIR's budget for research and development this year will be US\$650,000. "How can a country possibly prosper when its largest research organization receives less than the budget of a small science department at an average-sized Western university?" he asks.

It gets worse. The Medical Research Council has a research budget of just \$75,000. The Pakistan Science Foundation — the only source of government funds for university research — has only US\$250,000 to spend on research. The bulk of the Ministry of Science's US\$7 million research budget for this year will be taken up by a new science university in the twin-capital city of Rawalpindi (US\$1.75 million), the implementation of a national technology policy (US\$1.75 million) and the final tranche of students sent abroad for PhD degrees (US\$1.75 million).

Syeda T. K. Naim, a science policy analyst who headed a two-year review of sci-

Science in Pakistan

This supplement was written by Ehsan Masood, a staff writer on *Nature*. He would like to thank, in particular, the Pakistan Atomic Energy Commission, the Ministry of Science and Technology, the Pakistan Academy of Sciences and the staff at the High Commission for Pakistan in London. *Nature* would particularly like to thank those who generously gave up their time in aid of this project. A bibliography and list of references is available on request. Ehsan Masood's book on science policy in Pakistan is due to be published in 1997.



ence in Pakistan for the former prime minister Nawaz Sharif, says the shortage of funding has reached a critical level. "A leading institution of the Ministry of Science and Technology, for instance, has four cars but not enough typewriters, books or journals to carry out research work". Similarly, "a research scientist at a leading university, waited two years to buy a voltage stabilizer for his electron spin resonance machine, whereas funds were immediately available for the [departmental] chairman's office".

The shortage of funds, according to Naim, who has worked with the Pakistan Council for Science and Technology, an agency that publishes annual science indicators, has also dampened the enthusiasm of scientists. Indian scientists are more prolific than their Pakistani counterparts, despite being paid less. India has more

than 120,000 research scientists, compared with Pakistan's 8,500. Though, India's share of the world's published research fell from 3.16 per cent in 1981 to 2.34 per cent 1994.

There is, however, some good news. According to the Institute of Scientific Information in Philadelphia, there were 183 citations of Pakistan papers in all fields of science in 1981. This rose to 499 in 1994. Pakistan's share of world research authorship has doubled from 0.04 per cent in 1981 to 0.08 per cent in 1994.

Part of Pakistan's problem, says Naim, "is that scientists are chosen on political and personal connections, rather than merit". Among her recommendations to the prime minister — in what will probably be one of the few to be implemented before any subsequent change of government — she suggested introducing an element of competition in science by abolishing tenure for all new appointments.

But Pakistan's present science policymakers respond with some amusement to suggestions that the country somehow managed to stumble through five decades of existence without a coherent science policy or adequate funding. "This is patently not true", says Javed Jabbar, a former member of the Senate, Pakistan's upper house of parliament, and minister of science and technology in Benazir Bhutto's first administration. Jabbar, a colourful personality who also runs

one of Pakistan's leading advertising agencies, is widely regarded — even by his opponents — as an articulate science minister possessing, unusually for holders of this post, a considerable degree of influence.

Jabbar, who was instrumental in steering Pakistan's first national technology policy and allocating US\$70 million in government funds, says it is unfair to judge the state of the country's research by the tiny budgets and mediocre performance of universities and research councils "when two-thirds of Pakistan's research is done elsewhere".

Pakistan, according to the 1993 UNESCO *World Science Report* (the 1995 edition will be published at the end of this year) spends 1 per cent of its gross national product on science. This percentage is much lower than the Western average of 2 per cent. But it is higher than that of India (0.9 per cent), Brazil (0.4 per cent) or Turkey (0.7 per cent). In fact it exceeds that of two countries in the European Union, Greece (0.3 per cent) and Spain (0.7 per cent). And it is only fractionally lower than Ireland (1.1 per cent) and Italy (1.1 per cent)(see figure).

The 64-million-rupee question is: who spends the rest of Pakistan's not-insubstantial science budget? The biggest cheques for Pakistani science, it turns out, are signed not by the Ministry of Science and Technology, but by the Ministry of Agriculture and the Pakistan Atomic Energy Commission (PAEC), a sprawling organization whose title belies the scope of its work and the breadth of its influence. □

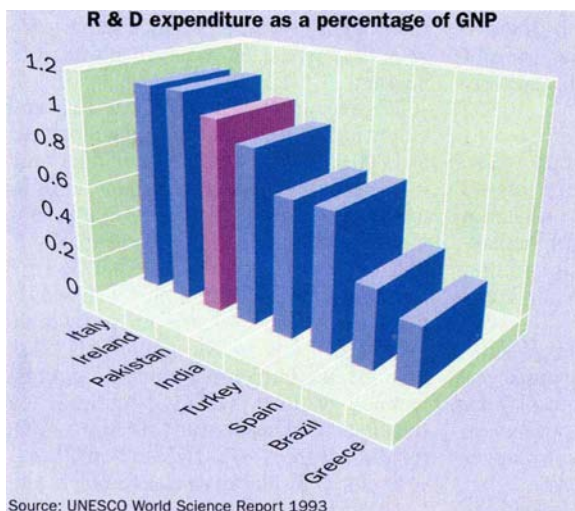
Fields of dreams

AGRICULTURE is the backbone of Pakistan's economy, contributing 38 per cent to the gross national product. Three-quarters of the population depends on agriculture for sustenance; it employs half the total workforce and constitutes 70 per cent of foreign earnings.

The Ministry of Agriculture is a powerful portfolio. And the Pakistan Agricultural Research Council (PARC), which reports to the ministry, has a research budget that represents 25 per cent of Pakistan's total spending on research and development. Agricultural research, together with the plant and animal sciences, constitutes almost 0.5 per cent of the world's published papers in these fields, and the numbers of citations per paper in agriculture is 59 per cent of the world's average in that field.

PARC's 'mission statement' is to develop and introduce high-yielding varieties of crops. It acts as a funding agency and conducts in-house research at the national agricultural research centre in Islamabad and 12 institutes throughout the country.

PARC's 50,000 affiliated scientists and technologists have scored some modest



successes — wheat production is up from 4 to 15 million tonnes; rice from 1 to 4 million tonnes; cotton from 0.3 to 1 million tonnes and sugar cane from 10 to 40 million tonnes. Other, more recent highlights include the development of disease-free potato seed, a high-yield variety of date-palm and the production of off-season tomatoes and cucumbers.

Despite such a massive investment, however, the overall picture remains grim. Pakistan's yields per hectare for most crops remain lower than those of many of its competitors. The yield of wheat per hectare is a third that of Mexico and a quarter that of France. Pakistan's rice yield is less than half that of Egypt and maize is less than a quarter that of the United States. Pakistan's sugarcane yield is 30 per cent that of India and less than half that of Egypt. Scientists also complain that farm-

ers are slow to exploit new varieties of seed and can be uncooperative if asked to change established methods of working.

Amir Muhammed, a former minister of agriculture and chairman of PARC from 1978 to 1990, concedes that ploughing more money into research is not the solution. "We need better management", says Muhammed, who now divides his time between running an international agriculture consultancy and holding the presidency of the Pakistan Academy of Sciences.

"We need less rhetoric from the politicians and clearly defined objectives, priorities and goals", he adds. "We know the soil, we understand the climate and our farmers possess centuries-old knowledge which you'll never find in books. We simply need to manage and process this information better, using the very latest tools at our disposal". □

Jewels in the atomic crown

THE Pakistan Atomic Energy Commission (PAEC) was formed in 1956, coinciding with the United Nations' Atoms for Peace conference. It has grown into a powerful department of state with a remit that covers agriculture, biotechnology and medicine as well as atomic energy. The commission reported to the minister of industries until 1972, when the then prime

disciplinary Pakistan Institute of Nuclear Science and Technology (PINSTECH). According to its director, N. M. Butt, PINSTECH contains the largest collection of trained, motivated and research-oriented scientists in the country. Housed in a building that is officially described as the Taj Mahal of science, PINSTECH was established in 1963 as a centre for research into nuclear physics, nuclear chemistry, materials science and the production and application of radioisotopes for use in medicine and industry. It is the largest industrial research laboratory in the entire Muslim world.

S M T Warsi/ PINSTECH

The institute, which is subject to full International Atomic Energy Agency (IAEA) safeguards, also contains a 30-year-old 5-MW research reactor of the 'swimming-pool' type. The reactor, known as PARR-1, was recently rebuilt, upgraded to 10 MW and converted

entirely by Pakistani engineers to operate on low enriched uranium. It is supplemented by a miniature neutron source reactor, PARR-2.

The institute developed reactor-grade fuel for the 137-MW Karachi nuclear power plant (KANUPP) when Canada stopped supplying fuel in 1976. PINSTECH is also in the midst of building, with the help of China and under IAEA supervision, a 300-MW pressurized water reactor at nearby Chashma.

But apart from PINSTECH, PAEC, particularly under the 19-year tenure (1972-91) of its longest-serving chairman, Munir Ahmed Khan, began to develop as an alternative centre for research in biotechnology and agriculture. The three

PAEC agriculture research centres in the provinces of Sindh, Punjab and the North-west Frontier have developed various varieties of cotton, rice, wheat and pulses.

A notable success was the development of a high-yield disease-resistant variety of cotton known as NIAB-78. Cotton production was, as a result, boosted by 6-10 million bales a year, bringing in additional revenue to farmers of between US\$1 & US\$1.5 billion. Nine PAEC medical centres provide radiotherapy facilities for 170,000 patients annually.

PAEC has recently branched out into biotechnology with the setting up last year of the National Institute of Biotechnology and Genetic Engineering. Munir Ahmad Khan, who has since retired to the international lecture circuit, reacted with bewilderment to a suggestion that PAEC should leave all non-nuclear research to the Ministry of Science. "The ministry of science and technology is too weak, vague and diffuse, while we are mission-oriented and get things done", he says. Similarly, he sees nothing objectionable in PAEC receiving its budget without a question in parliament. "It's less bureaucracy", he says. "And besides, nobody in parliament really understands science". □

Policy and politics

PAKISTAN has had four reviews of science policy since independence in 1947 when the country inherited a handful of research laboratories and just two universities, one of which was the University of the Punjab, built in 1882.

Two years into independence, the government established a food and agriculture research council and a department for scientific and industrial research reporting to the minister of industries. In 1953, this department became the Pakistan Council of Scientific and Industrial Research (PCSIR). The Pakistan Medical Research Council (PMRC) and the Pakistan Academy

Ayub Khan: Science-friendly president.

of Sciences were also formed that year. The Pakistan Atomic Energy Council — which later became the Pakistan Atomic Energy Commission — arrived in 1956.

The first attempt at coordinating science policy took place in 1959 with the convening of the National Science and Technology Commission following Pakistan's first military coup in 1958. The new president, Field Marshal Ayub Khan, today regarded as having made the greatest contribution to science and technology out of all of Pakistan's leaders, past and present, gathered around him some of Pakistan's most

Nuclear Mahal: PINSTECH is described as the Taj Mahal of science.

minister, Zulfikar Ali Bhutto, turned it into an autonomous department. PAEC's budget now comes directly from the presidential secretariat, bypassing both parliament and Pakistan's multi-tiered bureaucracy. Its chairman, Ishfaq Ahmad, is practically a science minister in his own right.

Most of PAEC's activities are public knowledge. But the size of the budget remains a secret. Basit Hasan's book *The Broken Ladder*, contains no references to PAEC's spending, and Naim says she was not allowed to look at PAEC's financial records for her two-year science evaluation. But authoritative sources put its budget at a third of Pakistan's spending on research and development.

The jewel in PAEC's crown is the multi-

prominent scientists. His personal science adviser was Abdus Salam, then a newly appointed professor of theoretical physics at Imperial College, London. The commission also included the chemist Salimuzzaman Siddiqui, who would later join Salam as, so far, Pakistan's only other fellow of the Royal Society.

The commissioners advised the president that scientific research should be coordinated by a single body and that the research councils needed to be headed by scientists. The exercise resulted in the setting up of an apex-shaped structure comprising a semi-autonomous National Science Council overseeing the work of research councils for agriculture, irrigation and flood control, atomic energy, defence, water, housing, medicine and the PCSIR.

The National Science Council enabled scientists to wield much influence and many regard the Ayub Khan years as the country's most scientifically productive. But the military counter-coup of 1969, followed by elections in 1971, led to the country's breakup when East Pakistan became Bangladesh.

The new prime minister of the truncated Pakistan was Zulfikar Ali Bhutto, foreign minister in Ayub Khan's cabinet, Benazir Bhutto's father and a populist socialist ideologue who campaigned on a promise to provide the poor with *roti, kapra, makan* — food, clothing and shelter.

Bhutto also had definite views about science. He summoned members of the National Science Council and told them that he intended to downgrade the body and place it under a newly created ministry of science. The Atomic Energy Commission was given autonomy and the two largest research councils, agriculture and defence, were relocated to their respective ministries.

Bhutto also created the Pakistan Science Foundation and the University Grants Commission as agencies to fund higher education and university research. But these measures were dismissed as mere trifles by scientists who were more concerned at their diminished influence over science policy under the Bhutto reforms.

The Bhutto structure is largely intact today. A National Science Policy document published in 1984 by the government of General Zia ul Haq made no further structural changes. Generous amounts of US and Middle Eastern aid, however, freed funds for the building of a new Islamic university and centres of excellence in electronics, silicon technology, biotechnology, oceanography and power.

The latest legislation, the publication of the National Technology Policy at the end of 1993 by the government of Benazir Bhutto, constitutes Pakistan's belated attempt at following the trail of the newly industrializing countries of South-East Asia, popularly known as the 'Asian tigers'.

Pakistan has allocated US\$230 million over five years for the policy's implementation. Its aim is to attract foreign, technology-based investment. Meanwhile, the PCSIR and the country's centres of excellence are to change gear, become more efficient and concentrate on international-standard wealth-creating activities. The government has also built a national science university at Rawalpindi and another is planned for Faisalabad.

One of the policy's architects, the former science minister Javed Jabbar, is confident that the exercise will reap rich

rewards, but scientists are less sure. Attaur-Rahman, director of the HEJ Research Institute of Chemistry at Karachi University, says the original Asian tigers, unlike Pakistan, enjoy high rates of literacy and a reasonably strong higher education sector.

Rahman also doubts whether Pakistan's research laboratories will rise to the challenge of 'frontier' research. "True applied research should lead to the development of new technology", he says. "In Pakistan, most laboratories carrying out 'applied research' are, in fact, reinventing the wheel." □

Abdus Salam and the shaping of science

IN 1974, Abdus Salam, not yet a Nobel prizewinner, in one of his final recommendations before resigning as the Pakistan prime minister's chief scientific adviser, suggested the convening of an annual forum where physical scientists from the developing world could meet and interact with their peers from the North.

Two years later, Salam led an international cast of physicists through the hills of northern Pakistan to attend the first three-week

Not the best of friends: Abdus Salam and Zulfikar Ali Bhutto.

International Summer College on Physics and Contemporary Needs at Nathiagali. In the subsequent two decades, 300 scientists, including six Nobel prizewinners have followed in Salam's footsteps and lectured on the frontiers and applications of physics to more than 3,000 participants from 72 countries.

The event is widely regarded as a successful example of North-South scientific cooperation. The organizers fittingly dedicated this year's twentieth anniversary meeting to Salam, the schoolteacher's son from a village in Punjab who shared the Nobel prize for physics in 1979 for his contribution to the theory of the unified weak and electromagnetic interaction between elementary particles and who, through the Third World Academy of Sciences and the International Centre for Theoretical Physics in Trieste, Italy, aspired to become a one-man bridge linking the fledgling science of the developing world with the advanced laboratories and research centres of the West.

Salam, nearing 70 and seriously ill, was unable to hear the generous public tributes to his life and work from, among others, his former students and peers, including Tom Kibble of Imperial College, London, as well as his intellectual successors, notably John Ellis of CERN (the European Laboratory for Particle Physics). There was even a belated

appreciation from Benazir Bhutto, conveyed by her representative, Shahnaz Wazir Ali.

But, then, Salam was also spared the comments of those in his country of birth who continue to take a less generous view of his achievements, particularly for science in his home country. Despite his many achievements, Salam remains a controversial figure in Pakistan. Hounded for his 'heretical' religious beliefs, criticized for

his bias towards theoretical physics and castigated for rejecting Pakistan in favour of Europe as his base of operations, he has never quite managed back home to match his doe-eyed image in the West.

Between 1961 and 1974, Salam, as the country's chief scientific adviser, commanded the attention of three successive Pakistani leaders, including the military president Field Marshal Ayub Khan and the elected prime minister Zulfikar Ali Bhutto. No other individual has held such an influential position for so long, before or since. Yet his critics allege that there is little outside the realm of nuclear and elementary particle physics to show for Salam's 13 years in the top science post. His period, they claim, was characterized by excessive idealism, a torrent of rhetorical statements and a failure to set and achieve realistic goals.

"Pakistan is not an efficient, developed country", says one of his peers. "It's a fact of life in this country that there is never going to be a massive injection of funds for science. But Salam never came to terms with this and always got angry and frustrated when the authorities rejected his larger-than-life schemes. Here, you have to make the best use of what you've got."

But Salam's friends and former students are keen to rally to his defence. At

S M T Warsi/PINSTECH

last month's Nathiagali meeting, several proudly recounted how Salam headhunted I. H. Usmani, a particle physicist turned bureaucrat, for the job of chairman of the Pakistan Atomic Energy Commission during its crucial formative years between 1960 and 1972. Together, the Salam-Usmani 'dream-team' planned and set up PAEC's flagship laboratory, the Pakistan Institute of Nuclear Science and Technology. The pair, according to Salam's biographer, Jagjit Singh, were also responsible for the purchase from Canada of a heavy-water CANDU-type 137-MW nuclear power plant for Karachi and a 5-MW research reactor for PINSTECH.

Salam is also credited with guiding the graduate physics programme at Quaid-i-Azam University in Islamabad and the University of the Punjab and establishing and serving as founding director of the Pakistan Space and Upper Atmosphere Research Commission (SUPARCO), not to mention the International Centre for Theoretical Physics and the Third World Academy of Sciences.

But such 'achievements' tend to draw criticism, not least from those who claim that Salam's successes turned Pakistan into a one-subject country: top of the class for theoretical and nuclear physics; but relegated to the remedial group for everything else. Wads of scarce funds, the critics allege, were lavished, on Salam's advice, to build and strengthen theoretical physics and the nuclear sciences, but at the expense of a balanced, coordinated university research policy.

Ishfaq Ahmad, the present chairman of PAEC, told during his own tribute how Salam arranged for 500 Pakistani scientists and engineers to go to the United Kingdom and the United States for training in nuclear science. He also recalled with some satisfaction how Salam ensured that PAEC staff were paid higher salaries than their counterparts in the Ministry of Science. But even Ishfaq, whose three decades with PAEC have turned him into a model of caution and diplomatic understatement, acknowledged that "we've had a lopsided development of science in the sense that the nuclear sciences are somewhat more advanced".

Pakistan's university physics departments are further monuments to Salam's influence. The physics departments at both Quaid-i-Azam University and the UK-built University of the Punjab concentrate research activity on theoretical physics, a legacy of Salam's involvement either as a lecturer at the institution itself or as supervisor to Pakistani students at Imperial College, London, who later returned to Pakistan to take up jobs at these universities.

Only Karachi University broke the tradition by developing expertise in solid-

The particle connection

THE Pakistan government is being urged to release funds to allow a team of physicists to join the hunt for the Higgs boson at CERN (European Laboratory for Particle Physics).

The government signed a protocol with CERN in December 1994 for a group of ten physicists from the post-graduate Quaid-i-Azam University in Islamabad to work on the CMS part of CERN's Large Hadron Collider. But the authorities have yet to sanction the US\$2 million needed to secure Pakistani participation in the project for ten years.

"This is an important opportunity", says Kamaluddin, chairman of the university's physics department. "It will greatly benefit scientific research and development in this country, while the manpower and expertise will, in return, also benefit CERN."

In June, Michel Della Negra, a CERN

official, travelled to Pakistan where he lobbied President Farooq Ahmed Leghari to sanction funding. "CERN needs the money", he says. "But we also want the brains."

Pakistani participation at CERN, says Kamaluddin, will help dispel the overwhelming sense of isolation that has gripped the country's science community. Pakistan is one of several countries forbidden for security reasons by both the United States and the United Kingdom from acquiring 'advanced' technology.

Universities, which often rely on imported equipment for research, seem to have been major casualties. Apart from preventing Pakistanis studying for PhDs in subjects such as lasers and high-energy physics, the prohibition has made more difficult imports of items for research such as computer hardware. □

state physics. Riaz Ahmad Hashmi, the departmental head, attributes this to the fact that the university was established in 1951, several years before the start of Salam's influence over national science policy.

Critics of Salam say his failure to make a bigger contribution to science in Pakistan also centres on his refusal to make Pakistan his permanent home. Salam was full-time professor of theoretical physics at Imperial College throughout his period as chief scientific adviser to the Pakistan government. In 1964, he added a third title as director of the newly established International Centre for Theoretical Physics in Trieste. Singh maintains that the spectre of intellectual death dissuaded Salam from settling down in Pakistan permanently. But this role as 'adviser from a distance', says Singh, ultimately diluted his influence.

But there is another reason for Salam's absence from the Pakistani science scene. Any lingering hopes that he might one day return were dashed in 1974, when Salam resigned his post as science adviser in protest at Prime Minister Zulfikar Ali Bhutto's decision to declare as non-Muslim the Ahmadiyyah religious sect of which Salam was an active member.

Ahmadiyyahs differ from Muslims in that they believe that the Prophet Mohammed was succeeded 1,300 years after his death by their founder, the nineteenth-century preacher from Punjab, Mirza Ghulam Ahmad, as the promised messiah or *mahdi*. An earlier meeting of the world's leading Islamic scholars gathered in Saudi Arabia had already declared them non-Muslim. It was left to

Bhutto to implement this decision.

According to Singh, relations between Salam and Bhutto never recovered. Bhutto did not always accept Salam's advice, particularly when it came to the prime minister's ambitious plans to turn Pakistan into a nuclear power. Salam, for his part, increasingly spent his time in Trieste.

Opinions about the full extent of Salam's views on Pakistan becoming a nuclear power remain divided. Although it is true that Salam always rejected the prime minister's offers to develop the bomb, Salam, in many other ways, had impeccable nuclear credentials.

He was an enthusiastic proponent of nuclear science in Pakistan, oversaw the development of PAEC and successfully lobbied for the purchase of the untried heavy-water CANDU-type nuclear power plant for Karachi instead of the light-water pressurized water reactor. The Canadians, realizing the plant's potential as a source of bomb-grade plutonium, cut off fuel supplies in the mid-1970s. Salam is also said to have advised Bhutto in 1973 to purchase the US\$300 million reprocessing plant from the French. The French, too, cancelled the order, under pressure from the United States, in 1978.

Salam continues to live the life of a self-exile. Despite his failing health, he remains a prolific writer. But visits to Pakistan are rare. Apart from a red-carpet reception by General Zia ul Haq following his receipt of the Nobel prize in 1979, this year's Nathiagali meeting was one of the few occasions in which the government publicly honoured its most acclaimed scientist. □

Daily life in the city of death

AFTER a week in Pakistan's pleasant, scenic capital city Islamabad, it is tempting to forget that the country's only port and commercial capital, Karachi, is in the grip of a complex and brutal civil war.

Thanks to state-controlled television, the day's death count often fails to make the headlines on the evening news. But Karachi

opportunities for jobs and education are fewer. There were few complaints in the beginning when the city's population was less than a million. But five decades and 10 million people later, many Mohajirs believe they were unfairly denied good jobs and a good quality education.

Parts of the city have become complete no-go areas, as angry young men target government officials, police and the paramilitary police, known as 'rangers'. The nature of the killings, too, has taken an horrific turn. Streets in the central district have become sniper alleys. And bodies turn up overnight on doorsteps, often diced and decapitated. All sides deny responsibility for the killings and, instead, accuse each other of terrorism. But the discovery of a network of MQM torture cells last year suggests the organization is more than just a political party.

Nuzhat Ahmed, professor of genetics at the University of Karachi and director of its Centre for Molecular Genetics, says daily life is becoming unbearable. Ahmed, an alumna of University College London, who lives in the staff town of the university campus in Karachi, says she is lucky to be able to work every day. "But other staff often take days off, particularly when there are strikes", she says. "I can't go out

Streets of strife: This year's death toll has exceeded one thousand.

is ablaze. A three-year standoff between the government and the MQM, a political party representing the *mohajirs*, or Urdu-speakers, a term of reference used for those who left homes in India and resettled in Karachi in 1947, has erupted into a bloody war between the government and MQM, between rival factions within the MQM and between the MQM and other nationalist groups. Last year, 1,800 people died. This year's count has passed 1,000.

Pakistan is divided into four provinces. The agricultural heartland of Punjab is home to the largest ethnic group, Punjabis, who make up 58 per cent of the population; Pathans, who live mainly in the mountainous North West Frontier province, constitute 13 per cent; Baluchistan, a large arid region to the southwest, but which has substantial deposits of natural gas, is home to a small number of Baluchis — 3 per cent. Sindhis, who live in the Southern province of Sindh — which includes Karachi — make up 12% of the country's population.

Each region has its own language, but English and Urdu are official languages. Before independence, Urdu was — and still is — the language of Muslims from North India. It is derived from Arabic, Persian and Sanskrit and written in a script close to Arabic.

Independence in 1947 witnessed the transfer of 6 million Muslims from India. The one million that settled in Karachi were predominantly Urdu-speakers.

The root of Karachi's problem, according to the MQM, is the hated 'quota' system, in which Karachi's 10 million *mohajirs* (literacy, 70 per cent) have to compete for less than 40 per cent of the city's government jobs and college and university places, regardless of their academic or professional suitability. The remaining places are reserved for those from rural parts of Sindh province where

or visit anyone. I haven't seen my relatives for months".

The violence, however, is affecting more than just commuting, says Ahmed. Her centre's budget is in danger of being cut. "It doesn't matter how committed we are as researchers", she says. "If we can't do the research, we won't get any money."

The university, which, until recently, witnessed unremitting violence between rival political groups, has since calmed down, owing partly to the authorities' decision to ban all student political activity. Armed paramilitary rangers keep a watchful eye on examinations also to prevent a recurrence of the university's one-time problem of examination malpractice.

Meanwhile, the government's response to the city's chaos has so far had little effect. It recently tried closing down six of the city's evening tabloid newspapers in an attempt to curb what it considers to be 'sensationalist' reporting. The city's mobile-telephone networks have also been switched off to disrupt communication channels.

But the MQM, whose leader, Altaf Hussain, directs the campaign from exile in London, commands unswerving loyalty from the majority of *mohajirs*. Its tactic of two-day weekly strikes has brought the city to a grinding halt and forced the government into talks, which recently broke down. Hussain has warned of a recurrence of the events of 1971, when East Pakistan broke away to form Bangladesh. □

The continuing question of the bomb

ABDUL Qadeer Khan, director of Pakistan's uranium enrichment laboratories at Kahuta near Islamabad, is that rare example of a scientist with a high public profile. A 60-year-old metallurgist, his popularity is on a par with that of pop stars and cricket idols. He is feted by crowds, is a sought-after lecturer and at one stage received fan-mail by the sackload.

Prime ministers from Zulfikar Ali Bhutto through General Zia ul Haq to Benazir Bhutto consider him a national asset; children want to follow in his footsteps; and women pen verses in his honour. The reasons for such adulation can be summed up in a verse sent to him by one of his fans: "Oh, Dr Abdul Qadeer Khan! You are an intelligent person of our nation who at last imprisoned the beams of the sun."

The question of whether Pakistan has the bomb might as well be as old as time itself. Western intelligence agencies take the view that Pakistan, which along with India and Israel has yet to sign the nuclear Non-Proliferation Treaty (NPT), possesses a nuclear device. But all Pakistan governments have insisted that their impressive nuclear facilities are "for peaceful purposes only". Pakistan, they argue, has no long-term fossil fuel reserves and so needs to develop nuclear energy. They also point

out that Pakistan, unlike India, has never tested a nuclear device. Successive leaders, moreover, have said they are ready to sign the NPT if India, whose own nuclear programme is more advanced than Pakistan's, follows suit. Pakistan and India, despite centuries of coexistence, today, are not friendly neighbours.



Abdul Qadeer Khan: we can go nuclear.

Khan says that the uranium enriched in his laboratories is only reactor-grade and not weapons-grade and that spent fuel from the Karachi power plant KANUPP as well as the two research reactors at the Pakistan Institute of Nuclear Science and Technology is subject to total International Atomic Energy Agency safeguards. He has given several newspaper interviews over the years denying that he has ever developed a nuclear device. But Khan — much to the government's discomfort — likes to tease and often mischievously ends an interview by adding: "we have the capability of going nuclear if the politicians so decide".

But whatever the outcome of the Kahuta project, Khan does not deny that it was born out of one Pakistan prime minister's desire to turn his country into a nuclear power. All of Pakistan's leaders have studiously allowed nuclear science in Pakistan to flourish, but none more so than Zulfikar Ali Bhutto.

As minister for fuel and natural resources in 1963, Bhutto laid the foundation stone for PINSTECH. He also negotiated the KANUPP deal and was prime minister when the plant went critical in 1971. Bhutto had also ordered a French reprocessing plant, which the French cancelled in 1978.

But it was in 1965, during the second war with India, that Bhutto, then Pakistan's foreign minister, came out with his most memorable remarks. "If India builds the bomb," he is reported to have said, "we will eat grass, leaves, even go hungry, but we will get one of our own. We have no alternative."

He got his chance in 1971. A month after taking office as prime minister after the fall of East Pakistan (Bangladesh), Bhutto gathered a select group of scientists who met in the city of Multan about 150 miles southwest of Lahore. He informed the group of his desire to see Pakistan build a bomb and that PAEC should be charged with organizing the project. According to Khalid Hassan, Bhutto's press secretary, the physicist Abdus Salam (see page 634) and I. H. Usmani, then chairman of the commission, tried to dissuade the prime minister. Bhutto, according to Hassan, decided to remove Usmani from his PAEC post and exiled him to the Ministry of Science and Technology.

Munir Ahmed Khan, fresh from a long stint with the International Atomic Energy Agency in Vienna, was installed as PAEC's chairman, a job he held for 19 years. But Munir Khan and PAEC are also believed to have stalled on Bhutto's plans until the arrival of Abdul Qadeer Khan.

After obtaining a PhD in metallurgy from the University of Leuven in Belgium and spending four years as a metallurgist at the Urenco enrichment plant in the Netherlands, Qadeer Khan arrived in Islamabad in May 1976 as an adviser to PAEC. Bhutto assigned him to work with Munir Khan on the uranium enrichment project. But the two soon fell out and, two months later, Qadeer Khan persuaded Bhutto to remove the project from PAEC's control. Bhutto agreed and handed control of the laboratory and its budget to Qadeer Khan who had direct access to the prime minister. This arrangement continues to this day, as does the uneasy relationship between Qadeer Khan and PAEC.

Bhutto was overthrown in a military coup in 1977, tried for ordering the murder of a political opponent, found guilty and hanged in April 1979. But his successor, General Zia ul Haq, sustained the project.

An ultracentrifuge was completed by 1981 and enriched uranium was produced in 1984.

If Pakistan does have the bomb, most observers believe it is unlikely to be found at Kahuta. For a scientist purporting to work on a 'top secret' project, Qadeer Khan has an unusually high profile. He is accessible, often in the public eye and rarely refuses interviews. He has also collaborated on an authorized biography, *Dr A. Q. Khan and the Islamic Bomb*, that goes

into considerable detail about the history and politics of the Kahuta laboratories and his often fraught relations with Munir Khan and the PAEC establishment.

But it is in this book that Qadeer Khan also acknowledges his ultimate debt to Bhutto. "If there was no Bhutto, there would have been no Kahuta plant", he says. And he adds: "had Zulfikar Ali Bhutto remained in power for some more time, Pakistan would have become a nuclear power". □

The high cost of learning

HIGHER education and university research in Pakistan have not had an easy half century. The numbers of universities have admittedly grown from two to 22 over the past 48 years. But this is still only one university for a population of six million people. Similarly, the nation's 75,000-strong student population, although 113 times larger than in 1947, represents just 3 per cent of the 17-24 age group.

University research is therefore considered to be something of a luxury, as evidenced by the size of the research budgets available and the number of PhDs produced. The combined income of all of Pakistan's universities, according to the Pakistan Council for Science and Technology, amounts to less than US\$40 million, out of which US\$1.6 million is spent on library materials, teaching laboratory equipment and research. Pakistani universities have awarded only 468 PhDs since 1947.

The physics department at the University of Karachi, the largest in the country with 700 undergraduates, funds its four undergraduate and five research laboratories on an annual research budget of US\$7,500. "You will probably laugh at this figure", says the department's chairman, Riaz Ahmed Hashmi. "We are forced to beg from other sources." The Pakistan Science Foundation, which traditionally funds university science, has said it is hard up.

The university's modest research programme in solid-state and condensed-matter physics survives instead on hand-outs

from the Pakistan Atomic Energy Commission, the Pakistan Council of Scientific and Industrial Research and a £320,000 grant over five years from the UK Foreign Office's Overseas Development Administration (ODA). But this money has now dried up and there is little prospect of receiving any more. "The ODA tells us it will fund projects with technical applications", Hashmi says. "But for us, this is difficult. We are ready to work with industry, but industry has not yet developed in a way as to be able to work with universities."

Hashmi says that successive governments have made great play with the idea that Pakistan can forge ahead technologically by promoting 'centres of excellence'. But the government, he says, should remember that it is throttling the one source that will feed these centres: university PhDs. Hashmi is therefore critical of government moves to build a centre of advanced physics at Islamabad based on the Trieste model. He believes that the centre will be irrelevant to applied physicists and could be used by politicians to argue against raising research budgets in individual university physics faculties.

Pakistan has more than 468 PhDs working in the country. But most of its 8,500 researchers obtained their doctorates abroad, under several schemes operating since the early 1960s, when more than a thousand Pakistanis were sent to Europe and the United States under the Commonwealth 'Colombo plan', and in 1985, during

Field	Total no. of papers	Share of world authorship (%)	Citations* per paper
Chemistry	508	0.13	0.23
Plant and animal	472	0.23	0.28
Physics	399	0.11	0.35
Agriculture	230	0.30	0.59
Clinical medicine	227	0.04	0.50
Ecology	107	0.17	0.47
Pharmacology	99	0.12	0.39
Engineering	93	0.04	0.28
Materials	92	0.09	0.28
Mathematics	25	0.05	0.94

Selected science indicators for Pakistan, 1990-1994

*Citations compared to world average = 1.00
Source: Institute for Scientific Information

General Zia's rule, when a further 1,500 scholarships for shortage subjects were reserved over 10 years. The last 100 from this scheme will depart next autumn, after which students will be encouraged to study for 'split' PhDs.

There is, however, one research institute — also within the University of Karachi — that continues to defy the country's research trend. The HEJ Research Institute of Chemistry, one of south Asia's finest, is a leading centre for natural product chemistry. It produces half of Pakistan's annual science PhDs and recently secured US\$10 million in funding for research from, among others, the German government and the US Department of Defense. Science in Pakistan — like the country's politics — is also heavily influenced by personalities. Whereas Abdus Salam shaped the nation's physics, Salimuzzaman Siddiqui, the institute's founding director between 1967 and 1990 and who died last year at the age of 97, was responsible for much of the centre's preeminence.

With the promised expansion in state university education still awaited, several

entrepreneurs and charitable trusts have moved ahead and built private universities. The trend began with the setting up of the elite Aga Khan University Hospital and Medical School in Karachi in the mid-1980s. The Lahore University of Management Sciences — with student fees of US\$4,000 a year — arrived soon after. Two years ago, the country's first private engineering university, the Ghulam Ishaq Khan University of Engineering Sciences and Technology (GIK) was set up at Topi near Islamabad.

GIK, which is named after a veteran Pakistani civil servant who later became the country's president, cost US\$30 million and contains the most advanced, and the most expensive, facilities of any Pakistani university or research laboratory. Fees are about US\$4,000 a year but the pro-rector promises that "no student will be turned away for the reason that he or she cannot afford to study at GIK". Most of the present batch of 300, however, pay full fees, providing a useful lesson that the best education — even in Pakistan — seems to be available only to those who can afford it. □

Planting the seeds of biotechnology

PAKISTAN may be top-heavy in the nuclear sciences, but signs are beginning to emerge of rapid development in other fields, notably biotechnology. The established centres for molecular biology at the universities of Punjab and Karachi were joined recently by the Centre for Advanced Molecular Biology, Lahore and last year by the inauguration of Pakistan's National Institute of Biotechnology and Genetic Engineering (NIBGE) in Pakistan's third largest city, Faisalabad.



Baby Bio: NIBGE is at the vanguard of biotech research.

The institute, established under the provisions of the 1984 science policy, exists, according to its director Kausar Malik, to provide biotechnology solutions for agriculture, medicine and industry. Just over a year old, it has already chalked up some impressive results.

Pakistan's textile industry, a significant portion of which lines the highway linking Faisalabad with Lahore, recently asked for help in detoxifying its effluent to meet new export guidelines. "The majority of the dyes turned out to be carcinogenic",

explains Malik. "We developed a range of microorganisms which are capable of not only degrading these compounds but which make the effluent colourless so it can be recycled."

Another key research area is the use of biotechnology in mineral extraction and fossil fuels. NIBGE is currently working on the bacterially mediated extraction of uranium and copper. Researchers are also working on the bioconversion of agricultural residues into methane gas and alcohol. In medicine, NIBGE staff have developed a DNA probe capable of detecting single cells of mycobacterium, the organism that causes tuberculosis.

But most of NIBGE's efforts are likely to be directed towards agriculture. Developing salt-tolerant crop varieties and nitrogen fixation are two important priorities. "Pakistan uses 30 per cent of its entire annual gas supply for the production of nitrogenous fertilizer", says Malik. The institute is therefore investigating various methods of biological nitrogen fixation, where genetically engineered microbes can convert atmospheric nitrogen to ammonia.

NIBGE's most impressive achievement, however, is the development of a transgenic leaf-curl virus-resistant type of cotton following the destruction of half of Pakistan's cotton crop from this virus for the past three years. Last year alone, Pakistan lost an estimated 4.4 million bales of cotton, valued at US\$1 billion.

Surprisingly, NIBGE does most of its work free of cost to the end-user — the government did not allocate any extra

money for leaf-curl research — although Malik contends that this state of affairs will not last long. NIBGE will, in future, undertake private projects through its newly established biotechnology company, Pakistan Innovative Biotechnology Services. The company has already won its first order, worth about US\$20,000, to produce a virus-free potato seed.

Malik says that Pakistan has until now had no tradition of contact between industry and the scientific community. Part of the reason, he says, is that "industry never had any confidence in scientists". But he acknowledges that "such an attitude has to change if this country is to harness the fruits of the technological revolution". □

Science city

THE Pakistan government has agreed to a proposal to convert part of the city of Faisalabad into the nation's first science enclave concentrating on research into agriculture and biotechnology.

The plan was put forward by Kausar Malik, director of the Faisalabad-based National Institute of Biotechnology and Genetic Engineering, and Anwar Nasim, executive secretary of the Pakistan Academy of Sciences. It has been included as part of the Faisalabad Masterplan, a government initiative to modernize this 100-year-old semi-rural city of three million people.



Anwar Nasim: Science city needs funds.

Faisalabad used to be known as Lyallpur, after the British governor who ruled before independence. A gothic-style clock tower in the town centre is a reminder of the city's colonial past. "Faisalabad is the ideal location for the science city as it is already home to four research institutes in agriculture and biotechnology as well as a university of agriculture", says Nasim.

The science enclave plan, he adds, envisages the addition of a university of science, a secondary school, industrial estate, congress centre and hospital. The provincial government of Punjab has agreed to build the science university and has allocated US\$2 million towards the rest of the project.

"We will need plenty more funds", says Nasim, a molecular biologist who recently returned to Pakistan after more than 20 years working for the National Research Council of Canada in Ottawa. He is particularly keen that Faisalabad's textile lobby should be involved. "I know people won't like me saying this, but we spend a lot of money on nuclear science. It's time that proper levels of funding were provided for other fields, too." □

Making a start on galactoaerhaeology?

The discovery last year of Sagittarius, a new dwarf galaxy, prompts a train of speculation about the degree to which the Galaxy has scavenged some of its bulk from the surroundings.

How exactly did the Galaxy to which the Sun belongs come into being? These days, of course, everybody knows that a question such as that must be heavily loaded with significance. Did our Galaxy form directly from primordial material (hydrogen, helium and some of the lightest elements) or did it benefit from an earlier recycling through stars, which would have created heavier elements? How long was the gap in time between the beginning of the Universe and the formation of the Galaxy? And given that some of the stars in galactic globular clusters appear to be at least 16 billion years old, how can so many cosmologists sleep easily in their beds at night when they know the latest estimates of Hubble's Constant give an age of the Universe only a little more than half that?

What follows is not, unfortunately, an attempt to answer those philosophical questions in the depth they demand, but rather is a consequence of the guilt evoked by a pile of unread astronomy journals accumulated since last March. Turning them into the library would get them out of the house, and cause no trouble in other ways, but seems a timorous course to follow. Why not browse through them first, to see if they have anything in particular to say. This, then, is the gleaning from half a dozen issues of the *Monthly Notices of the Royal Astronomical Society* (which now cutely belies its name by appearing every two weeks).

Readers of *Nature* know, of course, that galaxy formation is one of those central questions for cosmologists, like the meaning of truth for the world at large. Galaxies are supposed to form at points in the expanding Universe where there is an above-average fluctuation of the density of matter, which makes sense. In the days of innocence in cosmology, before 1981, there was a serious difficulty: the primordial density fluctuations would have been so great that only a few very large galaxies would have been formed. But then the inflationary Universe came along, supposing a smoothing expansion of the whole Universe by a factor of 10^{50} in the first few hours after the Big Bang, since when the conceptual difficulty has been that of inducing matter to condense into galaxies of any kind. (The famous 'missing mass', responsible for 90 or 95 per cent of the total mass

of the Universe, is the other product of inflation.)

It is therefore no surprise that a random set of issues of the *Monthly Notices* should be richly sprinkled with articles about galaxies and their formation. The place to start is with our own Galaxy, whose most prominent feature is a disk-shaped structure some 32,000 parsecs in diameter with the Sun placed roughly half-way from the centre. Who can fail to be intrigued by the case of the dwarf satellite of our own called Sagittarius, discovered only last year and believed then to be in the early stages of tidal disruption by our own (see R. A. Ibata, G. Gilmore & M. J. Irwin, *Nature* **370**, 194–196; 1995)?

What now emerges is that the disruption of Sagittarius is so great that the authors of the original discovery conclude that it must have made at least one transit of the galactic disk already, in the space of perhaps the past 600 million years. The same argument confirms that Sagittarius is one if the largest of the nine known dwarf galaxies trapped gravitationally by our own; it appears to be comparable in mass with that called Fornax and to be blessed with perhaps 150,000 stars, all of which now seem destined to end up in the galactic bulge. The two Magellanic Clouds, of course, are much larger, but are also showing signs of tidal stress.

The interesting question is not so much about the future as the past. How much of the Galaxy as it is now represents material gathered around the original density fluctuation and how much has subsequently been captured from the surroundings? It is bound to be a messy business, and might even be a fruitless one, to go hunting through the stars of the Galaxy's central bulge, looking for systematic patterns of velocity that would distinguish stars once belonging to now-extinguished dwarf galaxies, but there might be a little luck...

Where, in any case, did the dwarf galaxies come from? The standard explanation is that they were formed from intergalactic clouds either spontaneously or under the stimulus of the Galaxy's own gravitational field, but there is also an irrepressible body of opinion that these aggregations of stars are built around all-purpose black holes. Other schemes would have it that some at least of the dwarf galaxies have been split off

from the Magellanic Clouds by tidal interaction with the Galaxy, much as Comet Shoemaker-Levy was broken up into pieces last year by the tidal forces of Jupiter.

Working on the assumption that it should be possible to learn something of these tidal interactions from the distribution of stars in the known dwarf galaxies, Serge Demers (from the University of Montreal) and three colleagues from three different institutions (*Mon. Not. roy. Astr. Soc.* **274**, 491–498; 1995) have carried through a neat statistical analysis of the dwarf galaxies known as Leo I, Leo II, Fornax, Draco and Ursa Minor (all of which, like Sagittarius, are named after 'constellations' by convention).

Their objective is to look for non-random clustering of stars in the dwarf galaxies, using as a statistical criterion the test of whether groups of stars can be linked together by distances no greater than some arbitrary number. The outcome is disappointingly unsurprising; only in Ursa Minor is there a significant departure from randomness, a clump of about 78 stars (out of some 7,000) near the geometrical centre of the galaxy. Dutifully, the authors calculate that the mass of any black hole at the centre would be less than 10^6 solar masses; until more is known of the velocity of the stars in the dwarf galaxy as a whole, the necessity of a black hole to explain this clump is undecidable (and therefore irrelevant).

All this may be intriguing natural history, but it also sustains greater ambitions. Indeed, Ibata and Gilmore (both involved in the discovery of Sagittarius) have heroically collected spectra from 1,500 giant stars in the galactic bulge to construct a model of the dynamics of that region (*Mon. Not. roy. Astr. Soc.* **275**, 591–604 & 605–627; 1995). One outcome of that analysis is that the specific angular momentum of the bulge and of the halo that envelops it are essentially the same, but is markedly different from that of the disc. The model-builders will be delighted by all this, but what the rest of us wish to know is how much of the central bulge came with the rest of the Galaxy and how much has been scavenged from elsewhere. Archaeologists would prefer to go looking for remnants of earlier dwarf galaxies, or even for the origin of the globular clusters found predominantly in the parts of the halo near the bulge.

John Maddox

Ion channels lose the rhythm

Michael J. Welsh and Toshinori Hoshi

A THREE-YEAR-OLD girl began to have blackouts (syncopal attacks), which gradually decreased in frequency with age. Her electrocardiogram showed a prolonged Q-T interval. When she was 18 years old, she suddenly lost consciousness while running for a bus. When she was 19, she became quite emotional as a participant in a live television audience and died suddenly¹. With the report of Bennett *et al.* on page 683 of this issue² and other recent papers³⁻⁶, we are beginning to reach a molecular understanding of the disease that killed this young woman.

The congenital long-QT (LQT) syndrome is a relatively rare inherited disorder that causes syncope and sudden death due to ventricular arrhythmia. Typically, arrhythmias are precipitated by emotional

or physical stress in a young, otherwise healthy person. The diagnosis is usually based on a compatible clinical history, a prolonged Q-T interval on electrocardiogram — a measure of the duration of ventricular electrical activity — and family history.

Surprisingly, macroscopic currents from mutant channels were inactivated as fast or faster than wild-type currents. Instead of slowed inactivation, the current showed a small sustained, non-inactivating component.

Examination of single-channel openings revealed crucial abnormalities that could delay repolarization. In response to depolarization, a wild-type Na⁺ channel opens only once and is then inactivated. Most of the time, mutant Na⁺ channels were inactivated normally. However, on some occasions, mutant channels failed to be inactivated, producing the sustained current found in macroscopic measurements. This presumably prolongs the Q-T interval, predisposing to ventricular tachycardia.

Of course, many issues remain, some of which will be clarified by additional studies of patches containing a single channel. Behaviour of mutant Na⁺ channels should also be examined in mammalian cells because inactivation properties could vary, depending on cell type and the presence of other regulatory proteins. In addition, computer simulations incorporating detailed knowledge of normal and mutant channel function may generate explanations of how specific mutations can alter

cardiac action potential and predispose to arrhythmia.

Arrhythmias and syncope in LQT are often induced by sympathetic activation, so β -adrenergic antagonists are frequently prescribed. Why is this? Previous work has shown that Na⁺-channel activity is regulated by phosphorylation. It will be interesting to learn whether wild-type and mutant channels are differentially modulated when they are co-expressed with β -adrenergic receptors and exposed to β -agonists. Could reopening frequency be significantly altered in the mutant? Or perhaps a Na⁺ channel with abnormal gating predisposes to arrhythmias when adrenergic stimulation modulates other currents?

Recent work reports that mutations in a putative cardiac K⁺-channel gene are also associated with LQT³. Two studies^{5,6} have now shown that HERG is a voltage-dependent K⁺ channel, although the component of the cardiac K⁺ current resulting from HERG is uncertain.

What are the implications of these molecular insights for clinical disease? First, presymptomatic diagnosis should be possible, an important consideration when the first manifestation of disease may be sudden death. Second, when specific mechanisms causing arrhythmias are understood, therapeutic intervention might be tailored to specific genotypes and specific channel defects. Third, we may begin to discern relationships between the gene that is mutated (and distinct mutations within that gene), abnormal channel function and clinical manifestation. For example, in HERG six different mutations are reported: two deletion, three missense in conserved areas, and one splice-donor mutation. These mutations may alter function in different ways. Deletion and splice-donor mutations could decrease the absolute number of HERG K⁺ channels, thereby reducing K⁺ current. A non-functional missense mutation could have a dominant-negative effect, which in the presumed tetrameric HERG K⁺-channel complex would markedly reduce current. Or mutation could alter the properties of HERG channels, much as Bennett and colleagues have shown for SCN5A. If we learn that specific mutations give rise to different clinical and electrophysiological sequelae, the implications for understanding the cardiac action potential and for

Forms of long-QT syndrome

Syndrome	Chromosomal localization	Gene	Product	Inheritance
LQT1	11p15.5	?	?	dominant
LQT2	7q35-36	HERG	K ⁺ channel	dominant
LQT3	3p21-24	SCN5A	Na ⁺ channel	dominant
Sporadic	*	*	*	none
JLN†	?	?	?	recessive

Autosomal dominant LQT is also known as Romano-Ward syndrome.

*One case of sporadic LQT has been attributed to a mutation in the HERG gene³.

†JLN, Jervell-Lange-Nielson syndrome.

Clues to the molecular basis of LQT came when the disease was linked by positional cloning to three distinct loci (see table). Keating and colleagues³ looked for mutations in candidate genes that map to these loci. Two of the candidates turned out to be winners: HERG, a putative K⁺-channel gene which is related

- Schwartz, P. J., Periti, M. & Malliani, A. *Am. Heart J.* **89**, 378-390 (1975).
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management of the disease would be significant.

Finally, with the ability to identify mutations in these genes, we may discover previously unrecognized clinical phenotypes. Cystic fibrosis provides an example of how this can happen. Identification of mutations in the *CFTR* gene led to the surprising discovery that some of them cause a form of male infertility in the absence of other symptoms of cystic fibrosis. Note that *HERG* was originally cloned from a hippocampus complementary DNA library. Perhaps rigorous neurological testing might even reveal that carriers of *HERG* mutations have impaired or enhanced memory functions.

As information on LQT continues to emerge, we will surely uncover more sur-

prises about ion-channel function. But, of more importance, understanding the molecular basis of this inherited disorder will provide insight into more frequent arrhythmias. As William Harvey said in 1657: "...nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature, by careful investigation of cases of rarer forms of disease." □

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SOLAR PHYSICS

A slowly rotating core

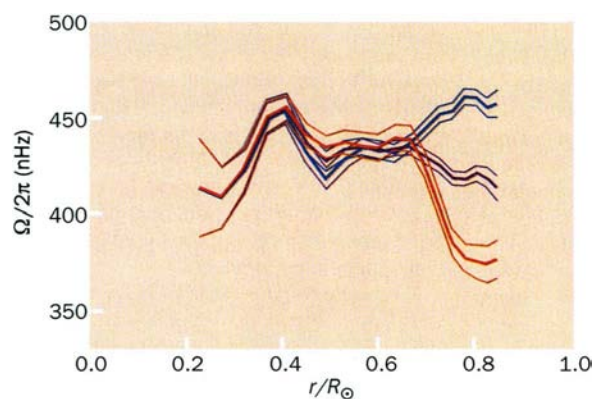
Jørgen Christensen-Dalsgaard

THE rotation rate of stars appears to decrease with increasing age, particularly for stars of relatively low mass such as the Sun¹. Stars are apparently born with rapid rotation, as a result of their formation through contraction of an initially slowly rotating gas cloud. The subsequent loss of angular momentum is thought to occur through a stellar wind, tied to magnetic field lines anchored in the exterior convection zone, where energy transport takes place through turbulent gas motion. Thus the immediate braking is on the convection zone.

The effect on the deeper parts depends on the transport of angular momentum through processes that are poorly understood, though it might be expected that this could have left regions in the solar interior rotating more rapidly than the surface. On page 669 of this issue², however, Elsworth *et al.* describe observations from the Birmingham Solar Oscillations Network (BiSON) which indicate that much of the Sun's deep interior is rotating at well below the surface rate.

The importance of this topic extends beyond understanding the rotational history of moderate-mass stars. Rapid solar internal rotation might induce sufficient oblateness to change the gravitational field outside the Sun and compromise tests of general relativity based on planetary orbits. Also, the transport of angular momentum from the solar interior to the convection zone could involve instabilities that might cause material mixing of the energy-generating core. This would change the evolution of the Sun and other similar stars, thereby, for example, causing ages estimated from observations of stellar clusters to be too small.

In recent years, information about the angular velocity of much of the Sun's interior has come from observations of solar five-minute oscillations. Their frequencies are perturbed by rotation; characterizing a mode by its degree l , radial order n and azimuthal order m (with m between $-l$ and l), the resulting cyclic frequency can be expressed as $\nu_{nl,m} = \nu_{nl,0} + m(\Omega)_{nl,m}/2\pi$.



Inferred solar rotation rate⁵, from inversion of data from the LOWL instrument developed at the High Altitude Observatory, as a function of normalized distance to the centre, at the equator (blue), latitude 45° (purple) and the pole (red). 1σ error levels are indicated by thin lines.

Here $(\Omega)_{nl,m}$ is essentially an average of the angular velocity over the region which the given mode occupies. So measurement of the rotational splitting $\nu_{nl,m} - \nu_{nl,0}$ provides a measure of rotation in a certain part of the Sun. By combining data for modes extending over different parts of the Sun we may infer the variation of the angular velocity with position.

Determination of the angular velocity becomes increasingly difficult with increasing depth, and inferences about the rota-

tion at distances from the centre of less than half the solar radius have been highly uncertain. Only low-degree modes, propagating nearly vertically, reach the central parts of the Sun and are sensitive to the core rotation. For these, the distance between the rotationally split frequency components, at most $2l(\Omega)$, becomes quite small. Probing the rotation of the energy-generating core requires observation of modes with $l = 1-3$, with a splitting of the order of a few μHz ; this is comparable to the natural width in frequency of the largest-amplitude modes, at frequencies near 3,000 μHz . Thus the split frequency components partly overlap, greatly complicating the observational task of separating them and determining the splitting. The results of such analyses have been correspondingly uncertain, although in several cases hinting at a small central region rotating at more than the surface rate^{3,4}.

Better determination of the splitting is possible at lower frequency, where the linewidth of the modes is smaller. Here, however, the oscillation amplitudes are small, making the identification of the modes very difficult. Nevertheless Elsworth *et al.*² now report the unambiguous determination of rotational splitting of many modes at frequencies below 2,000 μHz . This was achieved by combining data obtained from six stations over 32 months, thus reducing the noise level and confusion arising from gaps in the data. An example of the resulting power spectra is shown in Fig. 1c of the paper (page 670).

The results are fascinating: although there is substantial scatter in the data, almost all the splittings are below the value corresponding to the average rate of rotation in the convection zone. The outer layers of the Sun have a large effect on the average rotation rate as reflected in the splittings of even these low-degree modes, so this indicates that at least parts of the deep solar interior must rotate at well below the surface rate.

From analyses of previous data it has been possible to carry out inversions to determine the variation of angular velocity with position. An example⁵ is shown in the figure. It appears that throughout the convection zone rotation varies with latitude as on the solar surface, the poles rotating much more slowly than the equator. Near the base of the convection zone there is a transition, too sharp to be resolved with existing data, to essentially uniform rotation at a rate corresponding to an average of the rate in the convection zone.

The data of Elsworth *et al.* do not allow

an inversion to determine the variation of rotation in the core. However, the authors have carried out a least-squares fit to the data of various simple parametrizations of rotation, in most cases representing angular velocity as being constant over a few discrete shells and constraining rotation in the outer parts of the Sun to fit the results such as those shown in the figure. The values for the angular velocity depend on the assumed extent of the shells; but in all cases the results show regions rotating at well below the surface rate, confirming the initial impression (the results in the figure show a similar tendency, albeit with considerable uncertainty).

The physical origin of this behaviour is unclear. Elsworth *et al.* point out that because the minimum energy state is one of uniform rotation, the slowly rotating interior might be a transient state, possibly changing with solar cycle, or it might be caused by stresses resulting from material motion in the core or from magnetic fields. These possibilities will undoubtedly lead to extensive theoretical investigations.

Observations of solar internal rotation will soon be extended by the results from the Global Oscillation Network Group (GONG) project and the helioseismic instruments on the Solar and Heliospheric Observatory (SOHO) satellite to be launched late this year. Furthermore, continuation of the BiSON observations through at least one solar cycle, to test for the possible time-dependence of rotation, would be extremely valuable. The overall outcome may well be a significant change in our understanding of moderate-mass stars. □

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IMMUNOGENETICS

Female anti-male attack

Harald von Boehmer

THE immune system of females has been known to respond to male-specific transplantation (H-Y) antigens for some forty years, but the gene that encodes them has proved elusive. Now, on page 695 of this issue, Scott *et al.*¹ report that the *Smcy* (for selected mouse cDNA on Y chromosome)² gene is the origin of H-Y antigen, a result that was achieved through cosmid transfection and sequence analysis. Their conclusions are verified in an independent study by Wang *et al.*³, whose sequence of peptides isolated from class I molecules of the major histocompatibility complex (MHC) on the surface of cells from human males shows that they were derived from the human homologue of *Smcy*.

H-Y antigens were identified as a result of the rejection of male skin grafts by female mice from the same inbred mouse strain⁴. It was argued that genes on the Y chromosome control expression of H-Y antigens⁵ and that male-specific surface antigens could have a role in formation of the testes⁶, an intriguing idea that fell down when it was found that mice lacking H-Y antigens still developed testes⁷.

Of course in the early days of H-Y-specific immunity, the transport of intracellular peptides by MHC molecules to the cell surface was unheard of. The fact established later that cytolytic T cells recognize H-Y antigens in the context of MHC molecules⁸ was then consistent with the suggestion that male-specific peptides

could be degradation products of intracellular proteins that do not reach the cell surface in their entirety. It also became apparent that H-Y-specific immune responses could be generated in some mouse strains but not others, depending on whether or not these expressed 'permissive' MHC molecules^{9,10} that could present H-Y peptides. This fact, together with the notion that H-Y antigens are expressed on many different target cells, suggested that there could only be a limited variety of ubiquitously expressed, male-specific peptides.

Interestingly, the cytolytic response was not only controlled by permissive class I but also by permissive class II MHC genes, and it was found that H-Y-specific class-II-MHC-restricted T-helper cells and H-Y-specific class-I-restricted cytolytic T cells cooperated *in vivo* to bring about an effective immune response¹¹. In fact, H-Y-specific helper and cytolytic T-cell clones were isolated and used as tools to detect the surface expression of H-Y peptides. It is curious that the origin of male-specific peptides should have remained so

elusive, for three reasons — the H-Y response provided a neat illustration of MHC-restricted antigen recognition and thymic selection; the first MHC-restricted T-cell clone to be isolated was H-Y-specific (and specific for MHC alloantigens); and, finally, the first mice reported to be transgenic for the T-cell receptor expressed H-Y-specific receptors.

The identification by Scott *et al.*¹ and Wang *et al.*³ of the *Smcy* gene as the originator of H-Y peptides meets all expectations that could be derived from earlier studies of H-Y-specific immune responses: the *Smcy* gene is ubiquitously expressed in males only² and the male-specific peptides are encoded in regions of the *Smcy* genes that differ in their sequence from the *Smcx* gene¹², which is homologous to *Smcy* but located on the X chromosome and ubiquitously expressed in both females and males. This explains why the XX female can strike against an XY male, but the male cannot reciprocate.

It is probably not a mere coincidence that both the suspected murine H-Y peptide and the human peptide are encoded by *Smcy*, because so far this is the only ubiquitously expressed gene to have been identified on the segment of the Y chromosome that controls immunity to H-Y. Other genes identified in that region are the zinc-finger genes *Zfy-1* and *Zfy-2*, as well as the *Ube1-1* gene (for a review, see ref. 2), which are exclusively expressed in the testes.

The tracing of H-Y peptides back to the *Smcy* gene at last satisfactorily demystifies the H-Y-specific transplantation reaction which, in spite of its clinical relevance, represents a biologically irrelevant consequence of the ubiquitous expression of a gene whose location on the Y chromosome has been conserved over a hundred million years². What does remain a mystery, however, is the function of the *Smcy* gene during very early expression in embryogenesis and later on in a variety of tissues including the testes. Given the efficiency of transgenesis and gene targeting in the mouse, this should not remain a mystery for long. □

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Purpose of proton pathways

R. J. P. Williams

STEP by step, the features of energy capture and its transduction are being revealed. For the respiratory chain in bacterial membranes, this quest has culminated in the announcement by Michel and colleagues on page 660 of this issue¹ of the structure of the enzyme cytochrome oxidase, the last component of the chain.

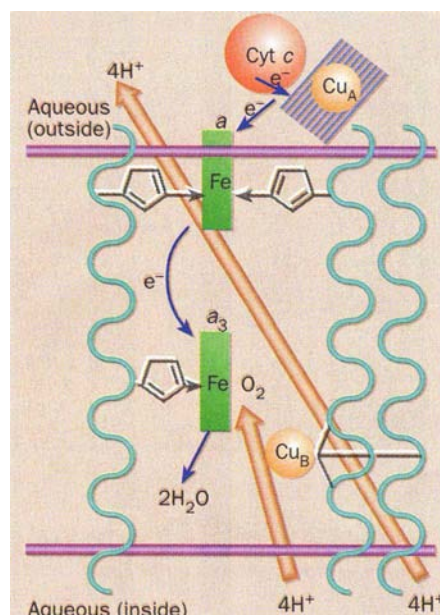
The respiratory chain harnesses the energy released by electrons derived from metabolic processes as they are transferred along the chain, using it to make a proton gradient and the ATP needed for activity inside the cell. Cytochrome oxidase is the enzyme that catalyses the last step in the respiratory chain, namely the transfer of electrons to molecular oxygen, which is converted to water, using and energizing protons in the process.

Michel and colleagues describe the structure of cytochrome oxidase from the soil bacterium *Paracoccus denitrificans*¹. Before turning to the significance of the structure, two points deserve to be highlighted. First, great skill has been shown in obtaining crystals of a transmembrane protein, achieved by using an antibody complex to stabilize the protein in a lattice. Second, the structure uncovers the manner in which the four membrane-bound subunits of cytochrome oxidase are held together. The functions of two of these are unknown, but subunits III and IV use their transmembrane helices to bind the active subunits I and II; subunit III also binds a lipid molecule. Hence this structure, together with that of the bacterial reaction centre, also solved by Michel and colleagues², opens up the prospect of reaching an understanding of (membrane) protein assembly.

Michel *et al.* discuss the function of cytochrome oxidase in the context of its structure. This is to take electrons from the penultimate electron carrier, cytochrome *c*, at a redox potential of around 250 mV, and to pass them to the dioxygen reaction site in the membrane, which has a redox potential of 800 mV (ref. 3). In the course of this reaction, which requires four protons, four additional protons (called pumped protons; ref. 4 and references therein) are energized by transfer across the membrane, so generating transduction to the full proton gradient. The figure summarizes what can be deduced from the structure in the light of earlier models.

The crystal structure shows that active subunits II and I are respectively a copper (Cu_A)-containing protein β -barrel and a helical transmembrane protein, which carries the two haem units and another copper (Cu_B) (see figure). Subunit II is an

unusual blue copper protein: it has two copper ions, not one, at the entatic site³ and its sequence is extended by two transmembrane helices which locate it in the membrane structure. It also has a suitable aqueous-phase binding site for cytochrome *c*. Subunit I has seven transmembrane helices and holds all three electron-transfer centres remarkably close together, with one, haem *a*, at less than 15 Å from Cu_A and 4.7 Å from haem *a*₃. The other two electron-transfer centres have a metal-to-metal distance of 5.2 Å. Note,



The proposed outline structure of cytochrome oxidase (modified from ref. 4). Thin arrows show the electron paths and the thick arrows the proton paths³. Histidine residues bound to iron are shown in grey; three more bind to Cu_B (see text).

however, that the Cu_B -coordinated ligands and the O_2 pocket are not well defined at this resolution. All three of these sites are much closer to the outside surface of the membrane than expected (see figure).

The electron-transfer rate, given the short distances and the large favourable driving forces⁵, must be much faster than the turnover of the enzyme at 10^3 per second. There is clearly no requirement for special through-bond electron transfer, but there are puzzles. Why have a Cu_A site with two copper atoms? Although this structure of the inhibited oxidized state delineates a possible site for dioxygen, does it give the correct site of binding in the reduced enzyme? A conformational change is probably an essential part of the redox reactions and the coupling of electron to proton movements. Long-range

conformational coupling is known in several helical haem proteins, for example cytochrome *c* and haemoglobin, but not in the β -sheet copper proteins⁶.

The nature of the two different proton paths (see figure) is still uncertain. Protons move in very short hops (smaller than 1 Å) and require rotational movements of many donor and acceptor groups to make the process continuous. Moreover, in this enzyme protons move along unidirectional paths. Michel and co-workers¹ point with confidence to a path for the protons for the $\text{O}_2 \rightarrow 2\text{H}_2\text{O}$ conversion, which stops at haem *a*₃ and Cu_B and starts from the inner side of the membrane (see Fig. 5b of ref. 1). These protons leave the O_2 -binding site as water and hence need a large (water) channel to escape.

The problem path is the one that involves pumping of protons across the membrane (one way only)³. These proton movements must be tightly coupled to redox changes and gated. They must not reach the dioxygen reduction site because this would uncouple their obligatory separate path. The use of the Cu_B histidine ligand as a swinging arm, as shown in Fig. 2 of ref. 1 and elsewhere, must surely fail in this respect. We have to conclude that the second proton-pumping path is still very uncertain and that we need several structures of different enzyme states to define this gated path, as Michel and colleagues¹ admit. It is salutary to remember the extensive work on different conformations that was necessary to follow protons in bacteriorhodopsin⁶.

The new work shows once again that, in membranes, biologically engineered proton paths dominate bioenergetics at least equally with electron paths. These pathways, required in quinone reactions of photoreaction centres⁷, in ATP synthetases⁸ and now in cytochrome oxidase¹, are clearly also demanded by the new outline structures of particle I of mitochondria⁹. Many of the descriptions of energy transduction in textbooks will need rewriting. □

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Dead trees tell tales

Stanley N. Williams

MOUNTAINS can be dangerous places, and on page 675 of this issue¹ Farrar and colleagues describe a hitherto neglected way in which they can kill. Their subject is Mammoth Mountain, in the east of California, a volcano that was last active several hundred years ago and is part of a larger volcanic basin known as the Long Valley caldera. Areas of dead and dying trees occur on Mammoth Mountain. The cause of death has been a mystery but Farrar *et al.* provide the answer — the trees are being killed by large volumes of carbon dioxide being emitted through the soil from magma deep within the mountain.

Long Valley is a busy recreational centre, Mammoth Mountain being a popular skiing resort because of the deep snow that accumulates on this eastern margin of the Sierra Nevada mountain chain. The dying trees near public camping areas, as well as the cases of near asphyxia in confined spaces that have occurred, have led to an upsurge in concern for "What might happen?". For all that the cause of the tree deaths is now known, the answer to that bigger question will remain elusive.

Events at Mammoth Mountain remind us that calderas such as Long Valley are not only big but complex, and that we don't understand how the voluminous eruptions that cause them actually begin. The reason is basically that we have no good historical examples of large-volume eruptions. Mount St Helens in Washington state was doing its thing in May 1980 when Long Valley began to shake, with several magnitude 5 tectonic earthquakes on the regional faults that cross the caldera. But the eruption that produced Long Valley some 760,000 years ago was about 500 km³ in volume, whereas that at Mount St Helens was only about 1 km³.

Elsewhere in the world, other calderas were almost simultaneously rumbling in the mid-1980s — Campi Flegrei, for example, which is next to the 11 million people of Naples, and Rabaul caldera, in which sits the important harbour of Rabaul in Papua New Guinea. Two neighbouring volcanoes finally erupted in Rabaul² during September–October 1994, after it had first shown signs of unrest in 1971 and significant activity in late 1983 through to mid-1985. Although the eruption was not of a scale even equal to that of Mount St Helens, it was enough to destroy many of the harbour buildings and homes, reminding us of how destructive caldera eruptions can be.

After the 1980 earthquakes in Long Valley, it was found that the caldera had

swollen, producing uplift of the central area by up to 75 cm, and the seismicity was interpreted to be related to magmatic resurgence beneath the area³. Eventually, those studying the deformation and seismicity produced a model of small dyke injections beneath the south-central moat of the large caldera⁴; and the soil gases mercury and radon were found to be anomalous by an order of magnitude over the area of the dyke, even though the dyke was still at a depth of 3 km (ref. 5).

Long Valley, however, is really a mixture of three different types and scales of



Study in brown at Mammoth Mountain.

volcanic feature (see map on page 675) — the caldera itself, the Inyo volcanic chain and Mammoth Mountain itself. We have all concentrated on the large-scale feature, with only a little work being done on the small young craters⁶ and almost none on Mammoth Mountain, even though it is a young volcano that has experienced an extended period of activity^{6,7}. A summary⁸ of the tectonic and magmatic processes of Long Valley and the associated 12 papers published in 1990 did not discuss the possible involvement of Mammoth Mountain in potential future eruptions. There were, to be fair, good reasons for that. The wilderness behind the mountain makes placement of seismic stations difficult; the snow means that studies of soil gas and deformation are terribly limited; and it was the one part of the caldera complex which seemed to be least active. So, the intense research and modelling of what was producing the activity at Long Valley rather ignored Mammoth Mountain.

Volcanic gases are useful as signals of reactivation of volcanoes because they are so mobile; for instance, Nevada del Ruiz

(Colombia), Redoubt (Alaska) and Pinatubo (Philippines) all showed visible changes in gas emissions for many months before there were significant seismic or deformation signals. Studies of the ideal stable gas, helium, have shown that gases may be moving through the volcanic edifice at several tens of metres a day⁸. Farrar and colleagues¹ find that carbon dioxide, the other not-highly-reactive gas, is not only killing trees but is advancing as a soil-gas 'halo' in advance of the obvious tree death or stress.

The gases are waving flags at scientists and, more importantly, the people who live or work near the activity. Sorey and others⁹ found strong gas evidence of magmatic activity beneath Mammoth Mountain, which was shown¹⁰ to be consistent with deformation once new stations had been added to Long Valley, with Mammoth Mountain as the centre of interest. (Our own investigations⁵ of soil gas, carried out in 1983, indicated that Mammoth Mountain was interesting but the unusually heavy snowfall of that year made a complete survey impossible and tricked us into not returning to the volcano.) All concerned should bear in mind that Mammoth Mountain did have small explosive events as recently as 500 ± 200 years ago⁶.

Etna and other volcanoes have also been studied for soil-gas emissions of carbon dioxide¹¹. The work on Etna, like the study by Farrar *et al.*, convincingly uses carbon isotopes to confirm that magma is the source of the diffuse soil gases. At Etna, it was found that emissions are of a magnitude that is almost equal to that of the gases that pour forth from the very persistently active, main-crater fumaroles and produce the large plumes. Volcanic activity is not so obvious at Mammoth Mountain, but Farrar *et al.* estimate that 1,200 tons of carbon dioxide are being released each day — that's just about equal to the flux found at other classically active volcanoes, such as Kilauea in Hawaii, which have open crater vents to facilitate gas release. An active magma source is sending out signals from deep beneath Mammoth Mountain, and we must give it much more attention. □

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Mixing fluids and solids

Rob Govers

PARTIAL differential equations are among the mathematical tools that scientists use to describe physical, chemical, biological and economic phenomena. Such tools are useful because they provide predictive capability for unknown conditions; engineers, for example, can use partial differential equations to test the performance of a car even before it has been built. The availability of powerful computers has made numerical solution of partial differential equations (the technical term for making a prediction) increasingly popular, especially one of the numerical techniques, the finite element method. A new finite element technique is described by Braun and Sambridge on page 655 of this issue¹, one which solves a long-standing problem — how to deal with solids and fluids at the same time.

Part of this problem is the frame of reference one has to choose to solve the equations of the motion of material, such as the Navier–Stokes equation. One choice is a reference frame which is fixed in space (a so-called Eulerian frame); a viewer in this frame of reference sees the material passing by, like watching water flow under a bridge. The space-fixed approach is very useful in calculations involving flow of gases (meteorology) and fluids (oceanography, hydrology). Its main disadvantage is that it is difficult to keep track of individual material points, which is needed for dealing with solids. This drawback can be overcome by using a frame of reference which is attached to the moving material (Lagrangian frame); an observer in this frame tracks the same material points all the time, as when focusing on a single car of a train as it passes. The material approach is widely used in engineering problems involving solids, but is generally impractical for the large and complex movements of fluids.

The choice of one of these two frames of reference is especially difficult in Earth science; on geological timescales, the viscosities of rocks span about ten orders of magnitude. The Earth's mantle, although solid, behaves like a fluid (on million-year timescales) which flows because of thermal convection. As a consequence, the space-fixed approach is well suited for numerical simulation of the Earth's man-

tle. The Earth's outer 100 km or so (the lithosphere) is much more viscous than the mantle, and the solid-like behaviour of this stiff shell determines the shape of the Earth's surface. Numerical solutions for the lithosphere have therefore most often been formulated in terms of a material-fixed reference frame. Difficulties arise where these regions meet — that is, where the lithosphere grades into the mantle, or lithospheric plates subduct into the mantle. Classically, the effects of the mantle on the lithosphere and vice versa are taken into account using *ad hoc* boundary conditions² or using a simplified representation of the other component³.

The method proposed by Braun and Sambridge overcomes these limitations because it can deal with fluids or gases and a solid at the same time. The natural element method they employ is a material-fixed approach, but it differs from the classic methods in that the governing equations are defined in terms of connections to neighbouring points and these connections are continuously updated. This keeps geometric elements from becoming too deformed for an accurate

solution of the partial differential equation, which was a snag with classic, material-fixed finite element methods. Braun and Sambridge cast their paper in a geodynamical context, but their method clearly has implications for a much broader community. For instance, studies of sloshing of liquids in flexible containers (hydroelasticity)⁴, hot forging⁵, solidification processes⁶ and cardiomechanics⁷ could all potentially benefit from this new finite element approach.

As with any new method, there are quite a few things which still have to be worked out. In many practical cases, sharp material boundaries, faults and element-based quantities, such as stress, will smear out. The step to three-dimensional applications needs to be made and, especially in these cases, the efficiency of the natural element method relative to classic finite element methods needs to be rigorously established. If these issues are adequately addressed, the natural element form of the finite element method may very well mature into a highly useful tool for solution of calculations involving fluids or gases and solids. □

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CLIMATE CHANGE

The evidence mounts up

Michael C. MacCracken

OUR present climate is unusually warm, and the pattern of warming over the past century strongly suggests an anthropogenic influence from greenhouse gases and sulphate aerosols. That was the message emerging from a week-long symposium examining climate variability over the past 1,000 years, which brought together results from a growing array of observational techniques, analyses of natural records and model results*.

Very precise measurements of the vertical profile of air temperature in boreholes drilled up to a few thousand metres deep indicate how the near-surface ground temperature has changed over the past few decades, over the past one or two centuries, and since the early part of this millennium (H. N. Pollack, Univ. Michigan). Papers were presented on results from Europe, North America, Africa, Asia, New Zealand and Australia; virtually all measurements indicate that there was an extended cool period a few centuries ago and that ground temperatures during the present century are on average

about a degree warmer than during the last century and, more importantly, than earlier this millennium.

Whereas borehole temperatures provide a direct but increasingly smoothed record, ice cores, tree rings and coral growth layers provide indirect, but year-by-year (and even season-by-season) estimates of the temperature and precipitation over much of the globe. Ice-core records provide information about volcanic eruptions, specifically the amount of sulphate aerosol injected (as deposited aerosols are trapped in the ice) and the cooling that it induced, which can be inferred from changes in oxygen isotope ratios (G. A. Zielinski, Univ. New Hampshire). Tree-ring evidence suggests that the coldest summers were 1601, 1641, 1669, 1699, 1783, 1816 and 1912 — with all but 1699 associated with known volcanic eruptions (P. D. Jones, Univ. East Anglia). The latest results suggest that the sulphate content of material ejected in the 1883 Krakatoa eruption was relatively low, leading to only minor global cooling.

Combined land and ocean records indicate that there has been a global warming of 0.3 to 0.6 K since the last century, albeit

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*Climate Variability and Forcing Over the Past Millennium, XXI General Assembly of the International Union of Geodesy and Geophysics, Boulder, Colorado, 3–8 July 1995.

with cooling in the North Atlantic and some parts of China and North America (N. Nicholls, Bureau of Meteorology Research Centre, Melbourne). Data on retreat of mountain glaciers from the tropics to high latitudes reinforce this evidence (M. F. Meier, Univ. Colorado). Although there is evidence that the seasonal cycle is also shifting towards earlier winters (D. J. Thomson, Bell Labs), there was considerable dispute about how certain we could be that greenhouse gas increases were accelerating the shift that is itself due to changes in the Earth's orbital elements.

Several investigators (G. C. Reid, National Oceanic and Atmospheric Administration, Colorado; D. V. Hoyt, Research and Data Systems, Maryland) reported statistical linkages between climate fluctuations and variations in solar irradiance, but the evidence that variations in the Sun's energy output can be large enough to cause the changes will be at best circumstantial until a plausible mechanism is quantified. A diagnostic model aiming to match the climate record of the past few centuries indicated that solar variations could only have had an important effect in the unlikely event that the cooling influence of sulphate aerosols is largely compensating for the warming influence of greenhouse gases. For the future, all indications are that the greenhouse gas effect will increasingly govern the behaviour of the climate (M. E. Schlesinger, Univ. Illinois).

Interestingly, the warming pattern since the mid-1970s looks rather like the footprint of El Niño events, although this does not explain the strong warming over parts of Eurasia (C. K. Folland, Hadley Centre, UK). These quasi-periodic events warm the eastern tropical Pacific Ocean, which sets off a chain of events that cool the central North Pacific, warm northwest Canada, increase precipitation in the southeastern United States and intensify the large-scale pressure pattern across the Pacific, North America and the North Atlantic Ocean that alters mid-latitude storm tracks. Does this finding mean that the recent warming is due primarily to an increased (but not understood) frequency of El Niño events, or is this the pattern by which greenhouse-gas-induced warming is becoming evident? We don't know. Climate models are only just starting to achieve the high resolution and verisimilitude needed to reproduce, although often not strongly enough, the observed El Niño signature. Some model simulations suggest that the warming pattern will be similar to a persistent El Niño, but that oscillations will continue, superimposed on the higher average temperature (G. A. Meehl, National Center for Atmospheric Research, Colorado).

A variety of model simulations for the nineteenth and twentieth centuries are being conducted. Simulations that couple

ocean, atmosphere and land surface aerosols show better agreement with the historical record if they include the increasing concentrations of both greenhouse gases and sulphate aerosols than if they take into account just greenhouse gases (J. F. B. Mitchell, Hadley Centre; U. Cubasch, Max-Planck-Institut für Meteorologie, Hamburg; see J. F. B. Mitchell *et al.* *Nature* **376**, 501–504; 1995). This is evident both in the records of the global average temperature and in the geographical patterns of changes in surface and tropospheric temperatures (B. D. Santer and K. E. Taylor, Lawrence Livermore National Lab.). The chief discrepancy found in analyses of the vertical temperature pattern is that the simulated warming extends up into the lower stratosphere whereas the observations show cooling in this region. This discrepancy is probably a result of not including the effects of stratospheric ozone depletion, which other modelling studies indicate causes cooling due to reduced absorption of solar and terrestrial radiation.

In addition to lowering projections of overall global warming, including sulphates in the simulations has regional effects, including reversing the projected intensification of the Asian summer monsoons found in greenhouse-gas-only calculations (Mitchell). This occurs because the high (but uncertain) projections of future sulphur emissions (mainly from energy generation) for eastern Europe, India and China create a cooling pall over southern Asia. Interestingly, the change in the Earth's orbit since 6,000 years ago had a radiative influence of similar character, and the modelled and observed result of the change is a reduction of the summer monsoon and the aridification of much of the Middle East and northern Africa (suggesting that the models are responding as would nature).

Another important issue is understanding the internal variability of the atmosphere–ocean–(glacier) system. The picture slowly emerging from observations seems to fit reasonably well (at least in some cases) with mechanisms of decadal to interdecadal variability found in simulations with coupled ocean–atmosphere models, which are becoming more realistic (U. Mikolajewicz, Max-Planck-Institut für Meteorologie).

In the vernacular popular in the United States today, one could say that the DNA (here for Distinguishing Natural and Anthropogenic) evidence is becoming quite compelling. Although greenhouse gases and aerosols are not yet convicted beyond all reasonable doubt, the case is becoming steadily stronger. □

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DAEDALUS

Holes within holes

DRILLING for oil, says Daedalus, is a triumph of misguided technology. There must be a better way than sinking an outsized rasp into the Earth on the end of kilometres of revolving pipe. He recalls the destructive 'cavitation' created in a violently stirred liquid. Tiny bubbles of vacuum are formed, whose subsequent collapse creates vast local pressure and shock. Cavities can erode ships' propellers and give bite to ultrasonic drills. The problem is to create them on a large enough scale.

Daedalus's scheme is to create normal gas-filled bubbles, and then remove the gas. Chemists of a certain age may remember the venerable 'fountain experiment', in which water rushes into a flask to dissolve a soluble gas, such as ammonia or hydrogen chloride. If you could suddenly insert a tiny bubble full of hydrogen chloride into water, the same thing would happen. The gas would dissolve almost instantly in the surrounding water, which would rush in from all sides. The bubble would collapse like a cavity. The smaller the bubble, the greater its surface-to-volume ratio, and the faster and more destructively it would collapse.

To generate the bubbles in the first place, Daedalus plans to make bubbles of a mixture of hydrogen and chlorine, and expose them to an intense sudden flash. This causes the gases to combine explosively to hydrogen chloride. After the initial detonating expansion, which may have useful destructive power of its own, the hydrogen chloride will dissolve and the bubble will collapse.

DREADCO engineers are now testing a pilot 'chemical cavitation' drilling rig. It has no moving parts. It is simply a system for dispersing a mixture of hydrogen and chlorine at the bottom of a water-filled hole, and a strobe flash-lamp for firing the bubbles at rapid intervals. The gases may be piped down to the drill, or generated *in situ* by electrolysis; either way, hundreds of kilowatts of power can be efficiently channelled into destructive cavitation. Hydrogen and oxygen, exploding to water vapour, may cavitate even more violently if ultraviolet flashes can be made intense enough to set them off.

The deeper the drill goes down, the higher the hydrostatic pressure at the bottom, the more forcefully the cavities will collapse, and the better everything gets. With no revolving shaft, it can be sent round corners, backed up to drill a side-arm on its own bore, and so on. Oil and gas prospectors should welcome the chemical cavitation drill with open arms. Its lack of moving parts may even appeal to dentists.

David Jones

Challenging times for bioinformatics

SIR—On July 28, 1995, the timely public release of the complete DNA sequence of *Haemophilus influenzae* Rd made the world of biology richer, in one day, by 1,830,137 characters of genetic information encoding a complete bacterial construction kit of 1,743 predicted protein genes¹. While marveling at the advances in sequencing technology one wonders how biologists will cope with the onslaught of data from this and other genome projects. To illustrate recent advances in informatics technology, we have analyzed the open reading frames of *H. influenzae* with the aim of adding as

much biological knowledge as possible to each predicted protein sequence. The computer runs were completed in three days using the GeneQuiz software system² which identifies likely protein functions from amino acid sequence information in a completely automated fashion. This resulted in a new set of 148 putative protein functions in *H. influenzae* (see Table) and confirmed most of the 1,007 already identified by TIGR¹.

A thorough and reliable analysis of several thousand protein sequences in one week requires more efficient tools than are currently used for routine data-

base searches. Our approach to high efficiency biosequence informatics mirrors that of high-throughput sequencing: to start with existing technology, add robotics, and focus on solutions for rate-limiting steps. In computerized sequence analysis, the analogue of a robot is a program which embodies key rules learned from a human expert and applies these at high speed. The GeneQuiz system was developed during the analysis of yeast chromosomes and mycoplasma sequences³. It combines such an expert module with existing methods, such as programs for similarity searches in sequence databases.

New protein functions in *H. influenzae* identified by automated sequence analysis.

0017	Formate acetyltransferase 1	0572	3D	PmpA peroxisomal membrane	1090	HelB. Cytochrome c biogenesis
0052	C4-dicarboxylate-binding periplasmic	#m		protein + Glutharedoxin	1091	HelC. Cytochrome c biogenesis
0053	3D RspB. Starvation sensing	0574	3D	Peptidyl-prolyl <i>cis-trans</i> isomerase	1092	Cytochrome c biogenesis
0121	UDP-N-acetylmuramate alanine ligase	0597		Hydrolase	1093	Pyoverdine transport. Cytochrome c biogenesis
0126	ABC transporter	0624	#m	Fmv+Fmu ATP-binding protein		
0131	Major ferric iron binding	0626		Large conductance mechanosensitive channel	1094	Ccl1/Ycf5. Cytochrome c biogenesis
0135	AraJ. Processing of arabinose polymers				1095	HelX. Cytochrome c biogenesis
0136	ThdF. Possible thiophene and furan oxidation	0634		NifH3. Nitrogen fixation	1104	Sialic acid transporter
0146	C4-dicarboxylate-binding periplasmic	0647		Mg(2+) transport ATPase C	1125	Transaldolase B
0164	#f NADH:ubiquinone oxidoreductase subunit A	0658		ABC transporter	1147	IIA-ntr. Nitrogen fixation.
0167	NADH:ubiquinone oxidoreductase	0718		VacJ. Intercellular spreading	1148	ABC transporter
0168	#f NADH:ubiquinone oxidoreductase	0723		TrkH. Potassium uptake	1153	3D Hypoxanthine phosphoribosyltransferase
0182	Glucokinase	0731	#f	Phenoxazinone synthase	1155	Activator of anaerobic ribonucleoside-triphosphate reductase
0183	Na(+)-linked D-alanine glycine permease	0740	3D	Phosphomannomutase		Thioredoxin
0184	Esterase D	0756		NlpD. Cell wall degradation	1165	Phosphate acetyltransferase
0258	#f Glycosyl transferase	0773		3-oxoadipate CoA-transferase subunit B	1203	ABC transporter
0270	NifH3. Nitrogen fixation	0831		Cell wall formation	1252	IS150 transposase
0281	Proline/betaine transporter PPII	0837		CpxR. Transcriptional regulator	1280	Nap57/cbf5/yhba family
0291	Mercuric transport. Periplasmic component	0852		Multidrug resistance protein B	1289	ABC transporter
0299	Prepilin peptidase dependent protein D	0858		Methenyl-THF synthetase	1300	Carbonic anhydrase
0323	Lactoylglutathione lyase	0860		SpoU. rRNA methylase	1301	3D Ferredoxin
0333	tRNA (uracil-5-)-methyltransferase	0864	3D	Elongation factor G	1309	Sodium/myo-inositol cotransporter
0336	Mog. Molybdopterin biosynthesis	0881		Octaprenyl-diphosphate synthase	1315	Phosphomannomutase
0345	3D YojB. 4Fe-4S iron sulphur centre, electron transfer	0906		Nitrogen fixation	1337	ABC transporter
0346	MauN. 4Fe-4S iron sulphur centre, electron transfer	0929		Glutathionylspermidine synthetase	1342	Bacterioferritin
0347	Cytochrome c	0934		NrfF. Cytochrome c biogenesis	1349	Transcriptional regulator
0350	AmpG. Permease in beta-lactamase induction	0935		HelX. Cytochrome c biogenesis	1364	Acyl carrier phosphodiesterase
0364	Penicillin-binding protein 5	0936		Ccl1. Cytochrome c biogenesis	1366	Zinc proteinase
0376	HesB. Nitrogen fixation cluster	0948		Virulence-associated protein B	1368	Exodeoxyribonuclease small subunit
0388	O-sialoglycoprotein endopeptidase	0955		Ttk. Transcriptional regulator	1437	Transketolase
0389	Slp. Outer membrane	0959		Chitobiase	1439	CycZ. Cytochrome biogenesis
0393	GTP-binding	0961		Histidine triad zinc binder	1454	3D Phosphomannomutase
0409	TagE. Cell wall degradation	0963		FAD synthetase	1476	Transcription repressor
0412	Drap deaminase	0964		MviN. Virulence factor	1546	DNA repair
0415	Thiamine biosynthesis	0965		30s ribosomal protein S20	1555	RtsX. Cell division
0417	Thiamine biosynthesis	0977		Cell filamentation	1590	DNA polymerase III subunits γ and τ
0418	Proline/betaine transporter PPII	0979		NifH3. Nitrogen regulation	1607	HemM. Glutamyl-tRNA dehydrogenase
0456	ATP-binding pyrimidine kinase	0988	3D	3-Isopropylmalate dehydratase	1648	Amidotransferase
0464	Ribose 5-phosphate isomerase A	1001		Sporulation	1652	Extracellular endopeptidase
0469	Histidinol dehydrogenase	1004		Peptidyl-prolyl <i>cis-trans</i> isomerase	1665	Pepsinogen A-4
0493	IS150 transposase	1007		LytB. Penicillin tolerance	1684	3D Polyferredoxin
0494	NapA. Acid phosphatase	1010		3-hydroxyisobutyrate dehydrogenase	1688	NADH:Ubiquinone oxidoreductase
0508	MenG. Menaquinone biosynthesis	1019		Thiamine-binding. Periplasmic	1693	ModA. Molybdate-binding periplasmic
0509	MenA. Menaquinone biosynthesis	1020		Transport system permease	1694	Molybdenum transport
0519	NupC. Nucleoside permease.	1021		ABC transporter	1695	N-acetylmannosamine transferase
0520	Activator of pyruvate formate-lyase 1	1027	3D	L-Xylulose Kinase	1696	Glycosyltransferase in succinoglycan biosynthesis
0565	Phosphoglycolate phosphatase, chromosomal	1028		C4-dicarboxylate-binding. Periplasmic		
		1031		Malate dehydrogenase	1698	Glycosyltransferase in lipopolysaccharide synthesis
		1032		Icfr family of transcriptional regulators	1721	IS904 transposase
		1043	3D	Ferredoxin	1723	Nitrogen fixation cluster, ATP-binding
		1070	#f	HrpA. ATP-dependent helicase	1730	Bifunctional urea amidolyase
		1089		ABC transporter		

The 1680 protein sequences were provided as of July 31, 1995, by the TIGR Internet server¹. Numbers are the gene identifiers in the HIBD database, where 234 means HIO234.#f: Indicate where two or more sequence fragments have been merged due to the identification of putative frameshifts. #m: Potentially incorrectly merged sequences (missing stop codon) are indicated by giving two functions separated by a '+'. 3D: Proteins for which significant sequence similarity was detected to a protein of known 3D structure allowing fairly accurate three-dimensional models to be built⁶. 18 (out of 148) of the new functions identified are the results of database matches with proteins that entered the databases between 16 May 1995 (the submission date of the TIGR analysis) and Aug 3, 1995. In addition we have classified an additional 55 new functions from database hits as 'tentative' and another 254 as 'marginal'. These results are accessible over the World Wide Web (<http://www.sander.embl-heidelberg.de/genequiz/haemophilus.html>) in the form of 'living' features which are automatically updated as new data arrives.

es, enhanced by frameshift detection, pattern searches, family analysis, structure prediction, knowledge navigation tools and information parsers.⁴

Proposed protein functions (see table and the TIGR table¹) are, for the most part, implied from the known functions of related proteins. Depending on the species, context and evolutionary distance, however, the similarity of function between related proteins varies substantially. The assigned functions should therefore be treated as plausible hypotheses (or "putative identification"¹), ranging from certain to approximate.

The 148 new functions include, among others, 65 enzymes, 16 transporters and 7 proteins involved in electron transfer. These have increased the overall assignment levels for *H. influenzae* to 9% - implied known 3D structure, 65% - clear functional assignment, 82% - clear database similarity hit. The distribution of functional roles, classified in nine classes similar to those of Riley⁵, is very similar to that of the currently available *Escherichia coli* sequences.

This analysis of *H. influenzae* protein sequences illustrates how automated computational sequence analysis can be rapid, exhaustive and accurate up to a level normally reached through extensive work of sequence analysis experts. Keeping pace with future advances in sequencing technology by imaginative informatics methods will be a continuing challenge.

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Late experience alters vision

SIR — A visual target is said to 'pop out' if it is detected quickly and without serial search. Targets that differ from distractors by the presence of primitive visual features (for example colour, orientation) pop out whereas targets that differ based on other features do not¹. Treisman and Gelade¹ proposed that primitive features are processed in specialized modules, called feature maps. This hypothesis is consistent with neuroscientific evidence for spatially segregated cortical areas specialized for processing such features².

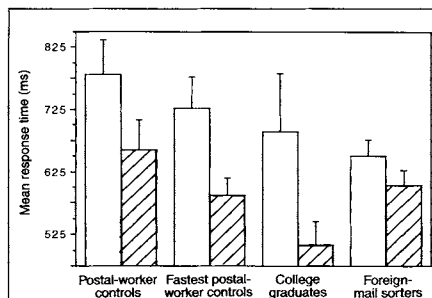
A similar pop-out effect occurs for letters and digits: a letter target is detected more efficiently among digits than among letters (and vice versa)³, suggesting that category information about alphanumeric stimuli is computed automatically and in parallel in much the same way as information about primitive visual features is processed. Consistent with this hypothesis, brain-damaged patients can be selectively impaired at letter recognition over number recognition⁴ and vice versa⁵, suggesting that letter and digit recognition are carried out by distinct maps. Recordings from human extrastriate visual cortex using chronically implanted electrodes also suggest segregation of letter and digit processing⁶.

Letter and digit recognition are not innate, so if the functional architecture of vision makes a distinction between letters and numbers, the environment must be helping to shape that architecture. How might this happen? The fact that neural learning is correlation-based suggests that correlations in the environment may

provide an answer. Stimuli within a category (letters) tend to occur together (in text) and are thus encoded in close temporal proximity, but stimuli between categories (letters and digits) co-occur less often. This statistical feature could interact with correlation-based learning in the brain to lead to maps for letter and digit recognition. We have implemented this hypothesis in a Hebbian neural network⁷ which, when presented with inputs that satisfy co-occurrence statistics, spontaneously self-organized to produce distinct letter and digit maps.

The co-occurrence hypothesis predicts a smaller category effect in subjects who regularly see letters and digits together. Such subjects should have less segregated maps and, as a result, letters should not pop out as much from digits. To test this prediction, we compared the effect in foreign-mail sorters at the Philadelphia Air Mail Facility with that in control subjects. Foreign-mail sorters spend roughly 4 hours per day processing Canadian zip codes in which letters and digits occur together (for example, M5S 1A4).

As predicted, foreign-mail sorters showed a reduced category effect compared with postal-worker controls, as measured both by the absolute difference in and ratio of response times in the letter-among-letters (LL) and letter-among-digits (LD) conditions (see figure). The sorters were, however, faster than controls, presumably because of their extensive experience with rapid tasks. To ensure that these results were not the result of a floor effect, we excluded the three slowest postal-worker controls (out of 16) for one analysis and used



Mean reaction times (with standard error bars) for correct responses on target-present trials in both letter-among-letter (LL; open bars) and letter-among-digit (LD; shaded bars) conditions for 16 postal-worker controls, the 13 fastest postal-worker controls, 8 college graduates and 10 foreign-mail sorters. All the foreign-mail sorters had been sorting foreign mail for at least 6 months before their participation. A computer presented subjects with a target letter followed by a circular array of letters or digits, and subjects were asked to press a key indicating whether or not the target was present. Details are based on experi-

ment 1 of ref. 13. *a*, Foreign-mail sorters compared with postal-worker controls. LD trials were reliably faster than LL trials ($t(25) = 4.367$, $P < 0.001$, one-tailed). Foreign-mail sorters showed a smaller alphanumeric category effect than did the postal-worker controls as measured both by the difference between the LL and LD conditions ($t(24) = 1.816$, $P < 0.05$, one-tailed) and by the LL/LD ratio ($t(24) = 1.864$, $P < 0.05$, one-tailed). *b*, Foreign-mail sorters against fastest postal-worker controls. LD trials were significantly faster than LL trials ($t(22) = 4.785$, $P < 0.001$, one-tailed) and the foreign-mail sorters showed a reduced category effect as measured by the LL-LD difference ($t(21) = 1.881$, $P < 0.05$, one-tailed) and by the LL/LD ratio ($t(21) = 2.060$, $P < 0.05$, one-tailed). *c*, Foreign-mail sorters against college graduates. Again, the LD condition was significantly faster than the LL condition ($t(17) = 3.208$, $P < 0.01$, one-tailed) and the foreign-mail sorters showed a reduced category effect as measured by the LL-LD difference ($t(16) = 2.271$, $P < 0.05$, one-tailed) and by the LL/LD ratio ($t(16) = 2.792$, $P < 0.01$, one-tailed).

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college graduates whose response times were faster for another. In both cases, the control group showed a larger category effect than the sorters, even though both groups were faster than the sorters in the LD condition. These crossover interactions eliminate any obvious interpretations based on scaling artefacts.

These results suggest that: (1) the alphanumeric category effect is modulated by environmental co-occurrence; (2) the effect is not just an artefact of physical differences between letters and digits; and (3) environmental statistics can influence the functional architecture of vision, even in adulthood. The co-occurrence hypothesis could also explain other examples of environmental influences on the functional architecture, for example, the neural segregation of handwriting compared with manual control tasks⁸ (and even script with print (M. Kinsbourne, personal communication) and upper case with lower case⁹); of melody recognition with the recognition of other sounds¹⁰; and of each language in bilinguals^{11,12}. These examples also involve influences on the functional architecture that are not innate. In keeping with the co-occurrence hypothesis, stimuli within these categories co-occur.

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Non-invasive bird tagging

SIR — Western European populations of the white stork (*Ciconia ciconia*) declined to near extinction in the 1970s¹. The populations were saved by keeping fledgling storks captive until sexual maturity (for about three years), thereby preventing their autumnal migration to Africa², where many deaths are thought to occur³. Once released, the former captives are provided with year-round food supple-

mentation and become settled (non-migratory). However, their fledgling offspring still depart in autumn at the same time as wild migratory storks and have been believed to migrate to Africa³, returning to breed only on reaching adulthood⁴. In the absence of firm information about such movements, the settling procedure has stimulated debate about its possible consequences.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

FIG 1. Implanting a tag under a stork's skin

Most knowledge about stork behaviour is based on ringing, but it is difficult and tedious to read rings of free-ranging storks. Satellite tracking of birds with attached radio transmitters has been used to follow migratory routes of some storks⁵, but in addition to the obvious disturbance of the bird by the transmitter, the transmitter battery lifetime limits the possible duration of tracking. To gain new insight into the behaviour of adult storks and their fledglings, we have used a different method to track the birds which, to our knowledge, has not previously been

used to investigate migrating birds.

An electronic tag (30 mm long, 3 mm in diameter, 0.8 g mass), permitting an automatic individual identification, was implanted under the stork's skin (Fig. 1). The tag is long-lasting because it does not require a battery. We have implanted tags in 290 storks without observing any tag-related infection or wound. Body condition can be assessed, as the birds in effect weigh themselves on scales coupled with tag-identification systems at feeding sites.

Our data show that feeding sites are used by all kinds of storks — immature and adult, free-ranging and settled. Settled adult storks of all ages (11–24 yr in our study) and both sexes vary seasonally in body mass, which is greatest in late January and lowest in early August (Fig. 2a). These results agree with the few data previously available from weighing storks directly⁶, but our method has the advantage of yielding many weighings without the stress of repeatedly handling the storks. Among 21 fledglings recorded for several weeks at Strasbourg (France) in late summer, just before the fledglings were supposed to depart to Africa for three years, five fledglings were again recorded at the same site during the next spring (Fig. 2b). In contrast to settled adult birds ($n=12$), which are 10% heavier in spring than in summer, those fledglings were 13% lighter in the spring.

Our data also show that the importance of feeding sites is not restricted to local storks. Two immature storks fitted with electronic tags at Planckendael (Belgium) were later recorded 400 km away at Strasbourg. The weight gain within a

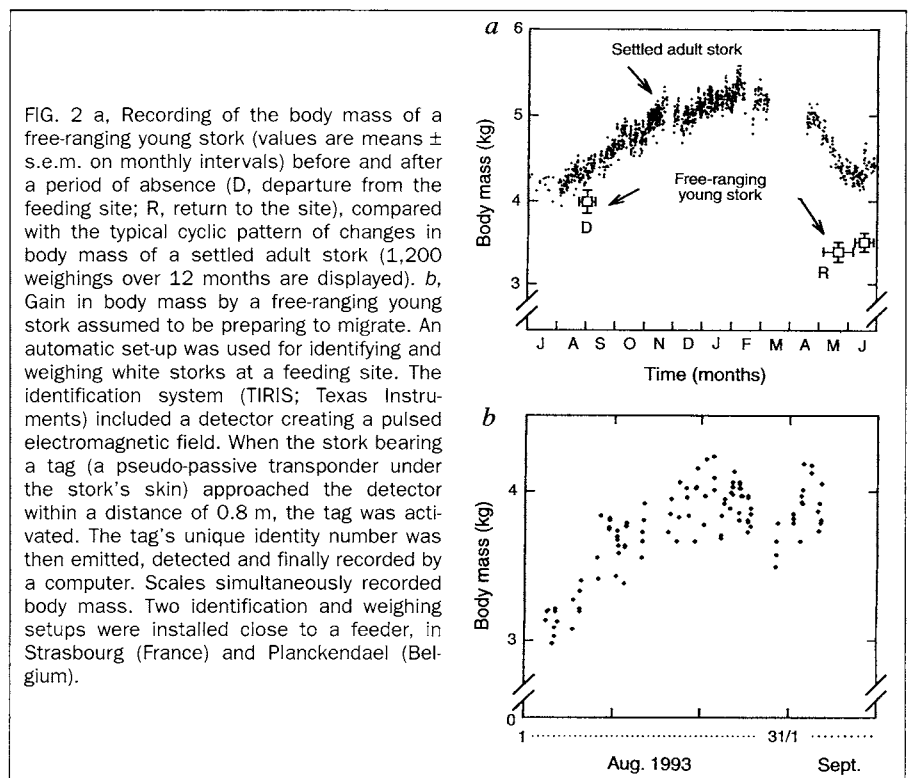


FIG. 2 a, Recording of the body mass of a free-ranging young stork (values are means $\pm$ s.e.m. on monthly intervals) before and after a period of absence (D, departure from the feeding site; R, return to the site), compared with the typical cyclic pattern of changes in body mass of a settled adult stork (1,200 weighings over 12 months are displayed). b, Gain in body mass by a free-ranging young stork assumed to be preparing to migrate. An automatic set-up was used for identifying and weighing white storks at a feeding site. The identification system (TIRIS; Texas Instruments) included a detector creating a pulsed electromagnetic field. When the stork bearing a tag (a pseudo-passive transponder under the stork's skin) approached the detector within a distance of 0.8 m, the tag was activated. The tag's unique identity number was then emitted, detected and finally recorded by a computer. Scales simultaneously recorded body mass. Two identification and weighing setups were installed close to a feeder, in Strasbourg (France) and Planckendael (Belgium).

month could be as high as 30% (Fig. 2).

Thus, it is now possible for the first time to identify individually and weigh migrating animals without handling them. In particular, we have learned that fledglings born to settled white storks do not necessarily migrate to Africa for three years. A network of identification sites for electronically tagged white storks could help us understand how human intervention to save endangered western European populations has changed the migration behaviour of this species.

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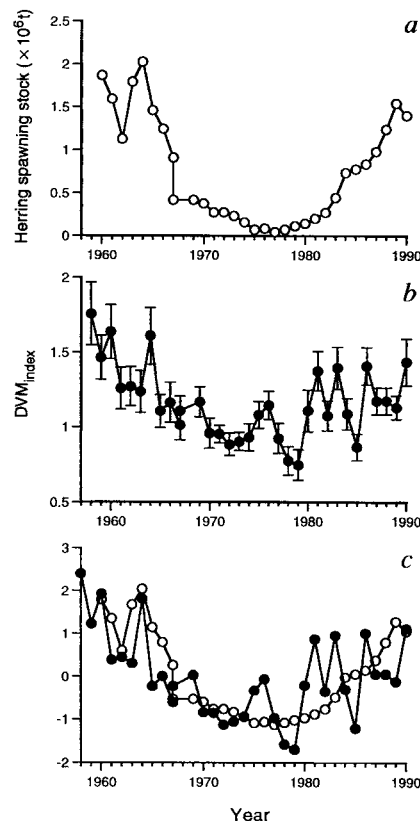
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Zooplankton avoidance activity

SIR — The structure of biological communities can show systematic long-term variations, but the effect of long-term changes in the abundance of one species or trophic group on other parts of the ecosystem is often poorly understood. In the North Sea, marked changes occurred between 1960 and 1990 in the size of the herring (*Clupea harengus*) stock¹. Data collected by the Continuous Plankton Recorder (CPR) survey² (CPR data were supplied by the Sir Alister Hardy Foundation for Ocean Science) show that during the same period there have been corresponding changes in the vertical migration behaviour of one of the most important prey species of adult herring, the copepod *Calanus finmarchicus*, with this species spending less time near the surface in the years when herring were most abundant.

Diel vertical migration (DVM) occurs widely in zooplankton communities, with a normal pattern of deeper daytime and shallower night-time occurrence. The normal DVM may serve to reduce the risk of mortality from visual predators such as fish³, being more marked in lakes when the abundance of planktivorous fish is high⁴, inducible in experimental enclosures by the introduction of planktivorous fish⁵, and, in localized areas, variable from year to year in response to variations in fish abundance⁶. Because long-term changes in fish stocks have been documented for very large geographical areas, it is conceivable that the normal DVM behaviour of zooplankton may change over correspondingly large spatial scales.

Stocks of herring in the North Sea declined from high levels in the early 1960s to low levels in the 1970s, before rising in the 1980s (*a* in the figure). Adult herring are planktivorous, with late copepodite stages of *C. finmarchicus* being a



a, The estimated spawning stock of herring in the North Sea during 1960–90. *b*, The normal DVM behaviour of C5–C6 *C. finmarchicus* in the North Sea (54–60° N, 3° W–10° E). For each year: $DVM_{index} = \text{antilog}[\text{mean log}(n_{night} + 1) - \text{mean log}(n_{day} + 1)]$, where n_{night} and n_{day} represent the number of specimens per sample for samples collected between midnight ± 6 h and midday ± 6 h, respectively. Error bars are ± 1 standard error. $DVM_{index} = 0.963 + 0.227$ (herring spawning-stock size in millions of tonnes), $F_{1,29} = 20.4$, $P < 0.01$, $r^2 = 0.41$. *c*, The two time series in *a* and *b* reduced to zero mean and unit variance to facilitate visual comparison.

major component of their diet⁷. Using the ratio of night: day abundance near the surface as an index of the extent of normal DVM (DVM_{index}), I found that the normal DVM behaviour of copepodite stages 5–6 (C5–C6) of *C. finmarchicus* co-varied with the abundance of herring (see figure). The implication is that in those years when herring were abundant, a smaller proportion of *C. finmarchicus* occurred near the surface during the day because of the increased risk of mortality. The unexplained variation in this relationship (*c* in the figure) may have reflected the low precision with which the DVM_{index} was estimated in each year.

To investigate this possibility, for various sizes of herring spawning stock randomly selected from the observed range, the DVM_{index} was predicted (using the equation given in the figure legend) and then from the observed distribution of errors (*b* in the figure) an error was randomly selected and added to each predicted DVM_{index} value. For 10,000 trials, the resulting r^2 value between the DVM_{index} and the herring spawning stock was 0.59. The observed r^2 value between these two time series (0.41) was therefore 70% of the maximum expected r^2 value given the precision with which the DVM_{index} was estimated, and hence no other factors need be invoked to explain much of the inter-annual variation in the normal DVM behaviour of *C. finmarchicus*.

These results suggest that the previously observed strong interaction between fish predation and zooplankton vertical migration in lakes and experimental enclosures also occurs on very large scales in the sea.

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Erratum

Following the publication of an exchange between Fowler *et al.* and Burton *et al.* (*Nature* **375**, 366–367; 1995), Fowler *et al.* have asked us to state that the final sentence of Burton *et al.*'s second paragraph was published without first being seen by Fowler *et al.*

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

Maritime sea changes

Janet Browne

HMS *Beagle*: The Story of Darwin's Ship. By Keith Stewart Thomson. Norton: 1995. Pp. 320. \$25, £18.95. To be published in the United Kingdom on 18 October.

ONLY a few ships in the history of science are famous enough for a full-scale biographical treatment in their own right. The *Astrolabe*, the *Challenger* and the *Endeavour* spring to mind; perhaps the *Roosevelt* or the *Trieste* or even the *Kon-Tiki*; and of course the *Beagle*, the ship that took Charles Darwin round the world from 1831 to 1836, providing him with experiences that ultimately intermeshed in his theory of evolution by natural selection.

If it were not for Darwin — and Darwin's later impact on the world of science — it is doubtful whether the *Beagle* would merit a book-length study. Yet the story of its life, both with and without Darwin, is utterly riveting. From the ship's birth (as a ten-gun brig at Woolwich naval dockyard) to death (sold as a hulk for scrap on the mudflats of the Thames), and with three extraordinary voyages in between, the tale reflects all the drama of overseas expansion in the nineteenth century at the height of Britain's imperial power. Keith Thomson's account could almost serve as a case study in the history of the British Navy during Queen Victoria's reign — a case study in the actual processes of exploration, with all the dangers of navigating uncharted waters, the careful surveys, the difficulties of finding supplies, the shipboard tensions, the routine plodding backwards and forwards from port to port, the accidents, the stupidities and the sheer sea-going skill involved. As Thomson rightly suggests, Darwin's footsteps on board comprised only a small part of this narrative. Such an altered perspective makes for an intriguingly refocused story in which the ship, and its company at large, take centre stage.

The *Beagle* began as an ordinary working vessel, no different from a hundred others in its class, built with an eye for adaptations to various purposes. Thomson provides a good deal of authoritative new information from the National Maritime Museum at Greenwich and elsewhere about the *Beagle*'s structure and its subsequent changes. Here his account supersedes all previous attempts to categorize the ship's technical history and will surely become standard.

But the ship was hardly used in its original form and was soon re-rigged as a barque (three masts instead of two) in order to become a survey ship. Its first assignment was to accompany the *Adventure*, commanded by Philip Parker King, in reconnoitring the eastern seaboard of

South America and establishing a British presence in the area before French or American forces. During this expedition the *Beagle* was sent south to survey Tierra del Fuego, at which point Captain Pringle Stokes was defeated by the gales, the poor handling of the ship, and scurvy among the crew. Stokes committed suicide; and the first lieutenant had to take the *Beagle* back to Rio de Janeiro on his own where Robert FitzRoy was quickly

consequence of FitzRoy's researches.

The ship's third and final voyage around Australia in 1837–43 has often been neglected but actually mirrors the last phase in the transition of the Victorian Navy from heroic, individual voyages of discovery to supplying the infrastructure of colonial life. Under John Clements Wickham, a veteran of the two previous voyages, and accompanied by many other officers and crewmen from the earlier voyages, the *Beagle* explored the north and northwest coasts of Australia in search of a safe shipping route between Sydney and the Indian Ocean. The men identified inland waterways, visited Port Essington (then just a few huts and a harbour), named Port Darwin and the River FitzRoy after their former shipmates, and delivered and

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The *Beagle* laid ashore for repairs in an estuary a few degrees north of the Strait of Magellan.

appointed to fill the vacancy. Back in Tierra del Fuego within the month, FitzRoy and his crew finished the survey, and found (and named) the Beagle Channel, a clear passage through the islands south of the Strait of Magellan.

When the voyage finished in 1830, King retired to farm in Australia and FitzRoy managed to persuade the Lords of the Admiralty that the South American survey still needed perfecting. Luckily, his requests coincided with the Hydrographer's wish to send a scientifically equipped surveying expedition round the world; and the *Beagle* was recommissioned and refitted for that purpose with all the most up-to-date instruments (including a lightning conductor), and FitzRoy was appointed as captain for a second journey south. This was the voyage that Darwin joined as a gentleman companion to the temperamental captain. Thomson describes such a well-worked episode in the *Beagle*'s career with commendable brevity and freshness; his interests range over the whole crew, not just Darwin and FitzRoy. He emphasizes the scientific results of the circumnavigation, many of them the

then rescued the future governor Sir George Grey not just once but twice from his almost fatal inland explorations.

After this, the *Beagle* was retired. American clippers dominated the oceans and the British context overseas was changing to settlement and commercial development. Thomson therefore escorts his ship, somewhat sadly, to a muddy berth in an Essex estuary for coastguard duty and thence to the firm of Murray and Trainer, scrap-dealers. This book is its only physical memorial; and Thomson is a fine memorialist. □

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■ The 'Darwin industry' continues apace with the appearance of two new books, one for specialists and one for general readers. James Moore's *The Darwin Legend* (Hodder and Stoughton, £6.99 (pbk)) is a fascinating piece of detective work that demolishes the myth of Darwin's deathbed conversion; and Michael White and John Gribbin's *Darwin: A Life in Science* (Simon and Schuster, £17.99) is a simplified yet readable biography.

Images of nature

Quentin Cronk

Green Imperialism: Colonial Expansion, Tropical Island Edens and the Origins of Environmentalism, 1600–1860. By Richard H. Grove. *Cambridge University Press*: 1995. Pp. 540. £45, \$64.95.

THE origins of a global environmental outlook can be traced back to 1600, according to this book, and our current perception of the biosphere has filtered down to us through a context of European colonial expansion. If these interesting claims are true, then it is inevitable that 'environmentalism' as a modern concept will carry with it a considerable amount of Eurocentric baggage. This baggage may place important and undesirable con-

cultural horizons through contact with new societies; and the environmental sensitivity of many island ecosystems — the idea of the poisoned and degraded Eden, the intellectual forerunner of which can be seen so clearly in Shakespeare's play *The Tempest*, where the idea of the island as an Eden is hedged about with ambiguities and conflicts, and where bewildering contact with indigenous society is represented by Caliban. Caliban's 'holistic' vision of the island — "I lov'd thee/And showed thee all the qualities o' th' isle./The fresh springs, brine pits, barren place, and fertile" — is rejected by the colonists. Gonzalo's "Here is everything advantageous to life" is quickly countered by Antonio's "True; save means to live".

As Grove argues at length and most cogently, no island has probably had more influence in provoking thought

past and continuing role of islands in environmental consciousness: they are minute simulacra of the Earth, isolated, vulnerable, yet small enough to be taken as a whole. Truly global thought is too difficult for the imagination. In the nineteenth century, tourists would hold up 'Claude glasses' to the scenery in order to reduce landscapes of incomprehensible scale to a pocket vision that could be held in the hand. The far oceanic islands have always been the Claude glasses of environmentalism.

Islands were also the first place where species extinction was made obvious. The essential condition for this is that species should be recognized as 'endemic' — if they occurred elsewhere then extinction in one place would be of little consequence. As early as 1805 (as Grove remarks) W. J. Burchell, seeing a falling rock split a gum tree (a member of the endemic genus *Commidendrum* in the Asteraceae), comments that he views "the demolition of one of these ancient gum trees with a superstitious concern, and the feeling of a fellow creature; for in all probability, unless St Helena be deserted, these trees would never again be suffered to attain so great an age, and (as this tree is peculiar to the island) this was sacrilegiously destroying the largest of the kind that would ever be in the world". Probably for the first time in human thought, sufficient knowledge of biodiversity and endemism, sufficient obviousness of environmental degradation and sufficient environmental and intellectual consciousness had come together to allow the articulation of a sentiment that is today a commonplace. That Burchell was on an island was clearly pivotal. The first concern about the disappearance of the native flora on St Helena, and attempts at conservation, date back to self-interest in the late seventeenth century when the island's council attempted to conserve timber supplies by fencing, grazing restrictions and later a planting law. In the early eighteenth century, when Governor Byfield was credited with saving an important timber tree, the redwood (*Trochetiopsis erythroxylon*), from extinction by planting seedlings in his garden, he was thinking of economics. Contrast this with W. Watson of the Royal Botanic Gardens at Kew, writing in 1888 about the extinction of the endemic *Commidendrum rotundifolium*: "As in point of structure it possesses peculiarities of its own, it is evident that the loss of such a tree is equivalent to the tearing out of the page of a record, or, to suit the Philistine mind, let us say the destruction of the page of a ledger". Nevertheless, the conversion of the Philistine mind still had a long way to go, and even on St Helena there was little practical done for species conservation until recent times.

Mary Evans

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A second biosphere — St Helena in about 1750, as depicted by J. van Ryne.

straints on global environmental consciousness, and must encourage careful examination of Richard Grove's thesis.

"This new kind of [global environmental] consciousness", he writes, "can now be observed to have arisen virtually simultaneously with the trade and territorial expansion of the Venetian, Dutch, English and French maritime powers. It was characterised by a connected and coherent intellectual evolution of ideas and concepts which had complex and yet identifiable roots in an Edenic and Orientalist search and in the encounters of a whole variety of innovative thinkers with the drastic ecological consequences of colonial rule and capitalist penetration." There are thus three elements to the intellectual change: the increase of territorial horizons (although the Spanish and Portuguese trading empires are excluded from this awakening intellectual development by Grove, even though they were wide-ranging in extent); the increase of

about the environment than St Helena in the South Atlantic Ocean, one of the world's most isolated islands. When St Helena was discovered by the Portuguese in 1501, they regarded it as a gift from God for the expansion of East India trade. Strangely, Providence had not provided any indigenous mammals for food (echoes of Antonio's "save means to live"), but this was immediately remedied by the introduction of goats. By 1588 they had multiplied so greatly that they formed "flocks near a mile long". As St Helena is only 10 miles long and 6 miles wide, even the most insensitive sailor could scarcely fail to notice the ensuing destruction in which two-thirds of the island's topsoil was washed out to sea and much of the vegetation irretrievably destroyed.

E. O. Wilson has called St Helena a second biosphere, isolated as if in space by great tracts of ocean from the rest of Earth, and in this concept resides the

One issue that Grove does not tackle is why these ideas met so much resistance for so long, and only bore fruit in cases of purely local initiatives for enlightened self-interest. Higher administration has tended to be at best apathetic to environmentalism, and at worst hostile. As late as 1938 Philip Gosse, St Helena's historian and champion wrote: "On both the steep sides of the Ridge the ruthless and rapacious flax growers have hacked down and grubbed up wild olive, tree ferns, cabbage trees, lobelia, and everything else which God planted there, in order to grow their flax, which would grow just as well in many other parts of the island. Let us hope that at this eleventh hour the Government of St Helena will forbid, once and forever, a single tree, shrub or fern to be destroyed in this wanton manner. The Ridge should be inviolate. It should be in the safe keeping of the Island for all time, and not one square

foot of it should belong to a private individual or company." The response was a profound silence.

Grove's book has performed a great service in pulling together so many disparate sources into a coherent whole in what is the most multidisciplinary of fields. Here the physician explorers who trained under the great botanist John Hope at Edinburgh jostle on the page with Philibert Commerson and Pierre Poivre, Khalif-al-Mansur, Thomas Hobbes and Robinson Crusoe in a vast historical tapestry that will raise more questions about the origins of environmentalism than it answers, but will be a stimulating, if controversial, source for any ecologist or environmentalist. □

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Technophobia then and now

David Edgerton

Rebels Against the Future: The Luddites and their War on the Industrial Revolution. By Kirkpatrick Sale. Addison-Wesley: 1995. Pp. 320. \$24.

Resistance to New Technology: Nuclear Power, Information Technology and Biotechnology. By Martin Bauer. Cambridge University Press: 1995. Pp. 422. £50, \$74.95.

Futile Progress: Technology's Empty Promise. By Ernest Braun. Earthscan: 1995. Pp. 218. £12.95, \$21 (pbk).

MAKING inventions and innovations is a wasteful activity. By their very nature, only a few will end up widely used. For the inventor and the innovator the failure of his or her brainchild to flourish in the harsh outside world may be attributed to many factors. One of the favourite explanations is 'resistance', which usually means that users and others are too stupid and unenlightened to understand the benefits of the new, or that those with vested interests do not want their old established position undercut. There is, it has been suggested, a 'cultural lag'. Sometimes this resistance is linked to the lack of scientific and technological education. Thus C. P. Snow dismissed British literary intellectuals (in fact a few novelists) as "natural luddites".

Indeed, the word 'Luddite' used in the general sense of resistance to new technology is an invention of the years after the Second World War; Snow was one of the first to use it. To be a Luddite was a thoroughly bad thing; resisting the march of technology was to renounce a better future. After the glory days of the long

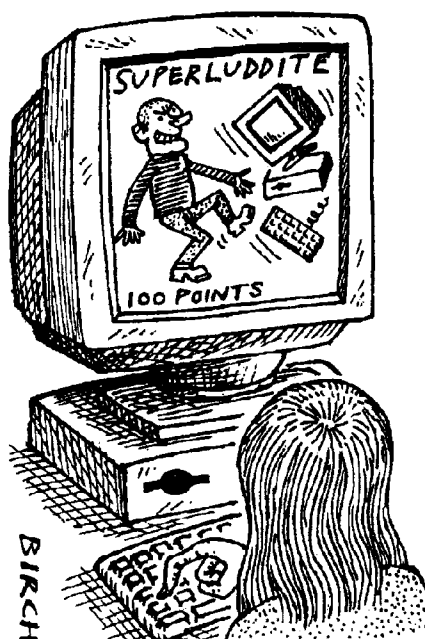
boom, such a view has come to be seen as dangerous and naive. Some argue that lack of resistance led to the acceptance of certain new technologies that defied economic logic and social needs. Nuclear power is perhaps a case in point. For others, the very concept of resistance was seen as an indicator of a culture that stigmatized any serious discussion of the merits of particular technologies.

Recent years have seen the reexamination of the story of the original Luddites. They were named after their nonexistent leader, often styled General Ned Ludd. General Ludd probably made his first appearance in Nottinghamshire in

November 1811; Kirkpatrick Sale estimates that between then and 1813, property to the value of £100,000 was destroyed in certain northern countries of England — about half in Nottinghamshire and the rest divided equally between Lancashire and Yorkshire. Perhaps 36 Luddites were killed; 24 were sentenced to death, 51 to transportation and 24 to prison. Indeed, the British government sent more troops to the affected counties than it did to the Peninsular War (1809–14). This great upsurge of machine-breaking was not the first or the last, but it was the most concentrated and spectacular. But machine-breaking was not a general phenomenon; it did not apply to all new machines or even really to new machines at all. As Adrian Randall shows in an article in *Resistance to New Technology*, an important factor was whether workers themselves would benefit from new machines. But the Luddite disturbances of 1811–13 were associated with high prices and high unemployment. Workers were resisting not new machines as such, but rather the new economic order that went with new machines and which was driving them into poverty. The resistance did not amount to much in the face of a state determined to let capitalism flourish, and with it the freedom of employers to use what machinery they wanted as they chose. Machine-breaking was made a capital offence by an unreformed, aristocratic state.

Sale's book is clearly sympathetic to the poverty-stricken followers of General Ludd, but also to more modern neo-Luddite intellectuals who call for a serious questioning of the benefits of new technology. Among these neo-Luddites we should include Ernest Braun. He calls for a steadier and more efficient rate of innovation, to give more time for assimilation, discussion and proper choice. He correctly points out that, at a national level, more spending on research and development does not lead to higher rates of economic growth. Particularly notable is his assessment of the British government's policies for science. He points to the inconsistency between free-market policies for the economy, and the dirigiste policies for science of industrial relevance. He calls instead for industry to be left alone to innovate and for state-funded research to be directed towards pure research, and also for research to be directed towards the non-industrial needs of the community. His is an interesting synthesis of green and free-market positions.

The volume edited by Martin Bauer consists of papers presented at a conference at the Science Museum in London. Under the rubric of 'resistance', many different stories are told from many perspectives. Some are studies of comparative diffusion; others of policies for



regulating particular technologies; some of public attitudes to certain new technologies. Although there are many interesting papers, they do not cohere as well as they might. But they do illustrate how misleading it is to try to understand the extent of usage of particular technologies using a concept of 'resistance', even if, as in this collection, 'resistance' is seen as inevitable and even healthy. It is significant that several contributors to the volume do not like the term 'resistance'.

As with the Luddites of old, it is not just new technology that is opposed — so is old technology; there are great differences in attitudes to different sorts of technology; and, furthermore, people do not usually oppose technology as such — they oppose particular consequences of technology. 'Resistance to new technology' is a misleading heading under which to discuss the usage of technology: the most obvious response of people to technology is to demand more, rather than to resist or oppose it. The most important limits to the diffusion of technology are economic ones. Cultures, far from lagging behind technology, demand new technologies and even imagine them before they are created. The creation of new technologies and the extent of their use are inevitably highly complex issues inextricably mixed up with economics, politics and society. But resistance to particular uses of particular machines is a universal and necessary feature of modern societies: we could not cope with everything that has ever been invented. □

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Gap filling

Douglas W. Burbank

Tectonics of Sedimentary Basins. Edited by Cathy J. Busby and Raymond V. Ingersoll. *Blackwell Science*: 1995. Pp. 579. £49.50, \$69.95.

ALTHOUGH there are many books by sedimentologists and stratigraphers on the depositional patterns within sedimentary basins, few volumes concentrate primarily on the tectonic setting, structural characteristics and tectonic evolution of these basins. *Tectonics of Sedimentary Basins* sets out to redress the balance. It contains contributions from many of the pioneers of modern studies of a tectonically broad array of basins. Some might argue that structural geologists would be better qualified to provide such a tectonic framework, but the authors have done an admirable job of synthesizing a truly

diverse literature in order to present clearly written and well illustrated summaries of 12 different tectonic classes of principal basin types. Because most of the chapters are by stratigraphers, there is a heightened awareness of the sensitive interplay of tectonics and sedimentation and the important role of the stratigraphic record in defining the details of tectonic histories.

Most of the chapters begin with a brief history of the ideas and nomenclature relevant to the basin type of interest, followed by a description of the main tectonic elements defining the basin setting. More structural detail and variations on the basic tectonic architecture are given before a discussion of specific examples from around the world. Sometimes the examples read more like a catalogue of global occurrences than intriguing illustrations of alternative themes.

On the other hand, several settings are treated by different authors from complementary vantage points. This enhances one's appreciation of the complexities and ambiguities of many well studied basins. Inevitably, the depositional systems within these basins are also described. But unlike many more stratigraphically oriented texts, the authors tend to concentrate on the tectonic controls exerted on depositional systems at a variety of temporal and spatial scales. Moreover, much of what is known about the tectonic history of these basins comes from stratigraphic study of their sedimentary fill. These stratigraphic descriptions therefore provide insight into the way in which such data are used to reconstruct tectonic events. The book is also enlivened by authors not afraid to express their opinions of earlier work or to examine controversial interpretations.

This is a wonderful reference book for anyone interested in sedimentary basins or who would profit from a bibliographic trove of more than 2,000 entries. The amount of attention given to tectonic settings varies from author to author, but even contributors who provide a less thorough geodynamical description offer lucid syntheses of the main elements and controls of basin-filling histories. There is some overlap among chapters because different authors deal with tectonically related settings; and a few of the figures reproduced from other studies are almost illegible. But overall this is an up-to-date and original compendium that embraces a spectrum of tectonic and settings in an uncommonly complete way. It will serve as a valuable reference well into the next decade. □

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New in paperback

Science on Trial: The Case for Evolution

by Douglas J. Futuyma. W. H. Freeman, £21.95 (pbk). This book first appeared in 1982, when the issue of whether both evolution and creationism should be taught in schools was in the news. The author aims "to show that creationism lacks both evidence and any claim to scientific respectability, and to place the conflict in its larger social, political, and educational context". A postscript lists important new developments bearing on the contents of each chapter.

In the Name of Eugenics: Genetics and the Uses of Human Heredity

by Daniel J. Kevles. Harvard University Press, \$16.95, £10.50. A classic account of the history of eugenics, now with a new preface.

Pride and a Daily Marathon

by Jonathan Cole. MIT Press, \$15, £11.95. A moving and informative story about a young man suddenly struck down by a rare neurological illness that deprived him of all sensation below the neck. The author, his doctor, brilliantly interweaves biography, science and philosophy. "A model of popular scientific writing" (John Marshall, *Nature* **356**, 636; 1992).

The Beak of the Finch: Evolution in Real Time

by Jonathan Weiner. Vintage, £8.99. A wonderfully detailed account of the work of Peter and Rosemary Grant, who for more than two decades have been studying the evolution of 'Darwin's finches' in the Galapagos Islands. "The style and presentation of this book are first class. It is one of the best pieces of science writing that I have read in a long while" (Jeremy Greenwood, *Nature* **371**, 27; 1994).

Correction

In the editing of Leonard Hayflick's recent review of two books on ageing (*Nature* **376**, 398; 1995), several changes were made that inadvertently introduced inaccuracies or altered the author's intended meaning. The second sentence should have read "We have learned how to eliminate most causes of youthful death, allowing most people to live long enough to experience ageing — a phenomenon that, teleologically speaking — we were never intended to see"; the second sentence of the last paragraph should have read "How long individuals will then survive depends on entropy, a process characterized by increased vulnerability to disease, predation and accidents"; and the author's correct address is Department of Anatomy, University of California at San Francisco, 36991 Greencroft Close, PO Box 89, The Sea Ranch, California 95497, USA. Our apologies to Professor Hayflick for these mistakes.

A numerical method for solving partial differential equations on highly irregular evolving grids

Jean Braun* & Malcolm Sambridge*†

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An efficient numerical method is described for solving partial differential equations in problems where traditional eulerian and lagrangian techniques fail. The approach makes use of the geometrical concept of 'natural neighbours', the properties of which make it suitable for solving problems involving large deformation and solid–fluid interactions on a deforming mesh, without the need for regriding. The approach can also be applied to high-order partial differential equations (such as the Navier–Stokes equation), even in cases where the evolving mesh is highly irregular.

NUMERICAL methods for solving partial differential equations (PDEs) require some form of spatial discretization, or mesh of nodes, at which the solution is specified. The nature of the mesh is an important factor in determining the accuracy and stability of the method as well as the type of PDE that can be solved. Straightforward methods, such as the finite-difference method¹ (FDM), are based on a regular spatial discretization, whereas more flexible techniques such as the finite-element method (FEM) divide the plane into triangles or rectangles, often with an irregular distribution². In many cases, the spatial mesh is made to change during the course of a calculation. An example is a 'self-adapting mesh' where nodes are added during the calculation to improve accuracy in a particular region, the position of which is determined by the solution itself³. The most difficult situation to handle is when the mesh is required to evolve with the solution⁴, that is, mesh nodes move during the calculation. An example is the case of advection in which the mesh nodes are 'attached' to a deforming medium. Although an evolving mesh allows for material properties to be accurately transported at the nodes, large displacements quickly result in severe mesh distortion which in turn increases numerical instability and restricts accuracy.

Here we show how the fundamental geometrical concept of 'natural neighbours'⁵ may be used to overcome these problems. The result is a new method which can be applied to problems where traditional eulerian and lagrangian techniques fail; for example, those involving large deformation, or fluid–solid interactions. The two essential features are the way in which both the mesh nodes and the connections between nodes are updated during the calculation to maintain an appropriate 'well-shaped' triangulation, and the use of 'natural neighbour' coordinates and their derivatives to interpolate smoothly between arbitrarily distributed nodes. We use a two-dimensional (2D) example of a sinking elasto-plastic plate in a linear viscosity fluid to illustrate these features, but all the theory described is equally valid in three dimensions. The crucial factor that makes the new approach possible is that the computational cost of all of the numerical algorithms increases at most linearly with the number of nodes.

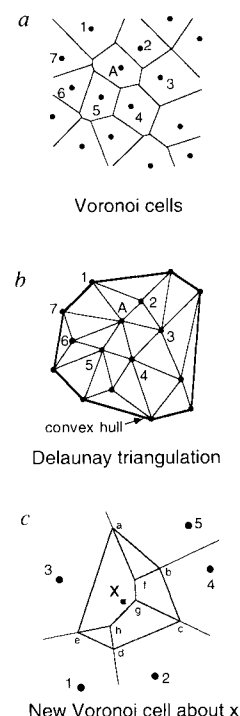
'Natural neighbour' coordinates

A unique and fundamental property of an arbitrarily distributed set of nodes in a plane (or in any dimension) is the set of 'natural neighbours' about each node. Natural neighbours are defined in

Fig. 1a and b. Any two nodes are said to be natural neighbours if their Voronoi cells⁶ have a common boundary, and the Voronoi cell about each node is the part of the plane which is closest to that node. Natural neighbours have some very interesting and useful properties. The number, and distribution, of neighbours about each node vary with the density of the nodes. Their distribution is a compromise between being 'close to' and 'surrounding' a node, and yet they are uniquely determined by the nodal distribution. By connecting each node to its neighbours we obtain the Delaunay triangulation⁷ which results in a 'well-shaped' set of triangles, that is, as near equilateral as possible.

FIG. 1 a, A set of randomly distributed nodes and their Voronoi cells in a plane.

The natural neighbours of node A are numbered 1–7. b, The Delaunay triangulation is obtained by connecting all pairs of natural neighbours together. The Voronoi cells are unique in any number of dimensions and their boundaries consist of the perpendicular bisectors between points. The Delaunay triangulation is unique unless four neighbours lie on a circle (or five on a sphere in three dimensions). c, A test point x and its five natural neighbours. The shaded area is the Voronoi cell about x . This first-order Voronoi cell can be divided into five second-order Voronoi cells. Each second-order Voronoi cell contains that part of the plane which is closest to x and second closest to one of its neighbours. Natural-neighbour coordinates, $N_i(x)$, are defined as the ratio of the area of the second- to first-order Voronoi cells. For example, $N_3(x)$ is equal to the area of the polygon (a, f, g, h, e) divided by the area of the polygon (a, b, c, d, e). Highly efficient 'local' algorithms have been found for calculating Delaunay triangulations and natural-neighbour coordinates in two dimensions^{8,13,15}. Sambridge et al.¹⁵ also present analytical expressions for the derivatives of $N_i(x)$ with respect to the components of x in two dimensions. Recently, we have implemented a different algorithm for calculating natural-neighbour coordinates and derivatives in three or more dimensions (see Box 1).



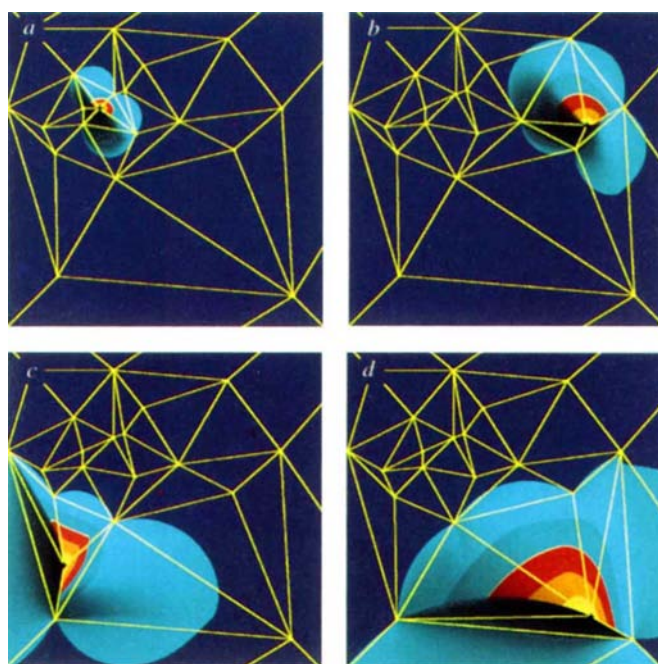


FIG. 2 The natural-neighbour influence function, $N_i(\mathbf{x})$, associated with a node i at various locations within a set of fixed nodes irregularly distributed in the plane. The influence function is displayed as an artificially illuminated surface with the height proportional to the value of the trial function about the node i marked by a cross (one at the node; zero at all other nodes). The surface is divided into five contours from yellow to blue; each band represents an interval of 0.2 in the value of the trial function. The surface shows how the node influences its surrounding region and that its shape is solely dictated by the distribution of nodes: it expands in regions of low nodal density (a) and shrinks in regions of high nodal density (b–d). An example of a 3D natural-neighbour influence function is shown in Figs. 3 and 4.

Efficient methods have been developed to calculate Delaunay triangulations (and the dual Voronoi diagram) in two and three dimensions^{8–10}. To make the Delaunay mesh useful in solving PDEs an interpolation method is required to estimate the solution (and its derivatives) between the nodes. A simple linear interpolation could be used¹¹ but this would restrict the approach to low-order PDEs. We use natural-neighbour

FIG. 3 Examples of the natural-neighbour influence function in three dimensions, calculated with the new method described in Box 1. Top left, a ray-traced image of a central node (purple) connected to its 12 natural-neighbour nodes (yellow). The connections between the neighbours are marked with a thin pipe (pink), while the connections between the central node and its neighbours are marked with a thicker pipe (green). Together they form 20 Delaunay tetrahedra, all of which have the central node at a vertex. The network shown is only a subset of a larger number of surrounding nodes distributed randomly in three dimensions. Top right, a surface on which the natural-neighbour coordinate with respect to the central node is a constant ($N(\mathbf{x}) = 0.1$). The value of the natural-neighbour coordinate represents the weight given to that node in natural-neighbour interpolation. Two other isosurfaces, at values of 0.05 and 0.0000001, are shown in the bottom left and bottom right panels, respectively. Notice how each surface is smooth. The smoothness properties of natural-neighbour interpolation arises directly from the continuity of this influence function. When the natural-neighbour coordinate is equal to zero, the isosurface consists of the union of 20 spheres (see Fig. 4). Each of these spheres passes through the four vertices of one of the Delaunay tetrahedra connected to the central node.

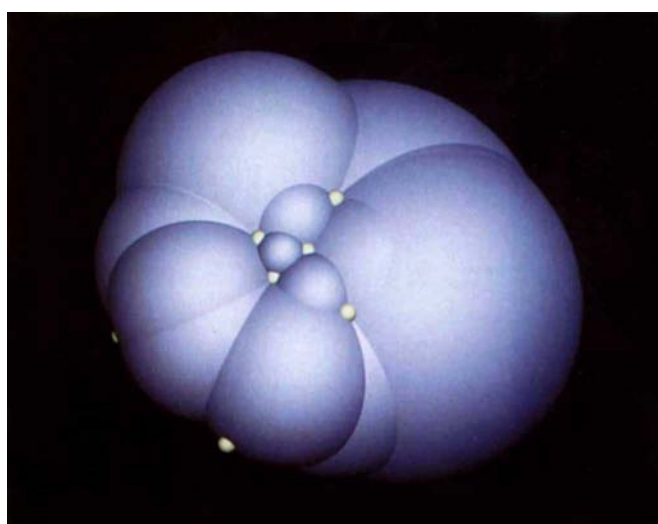


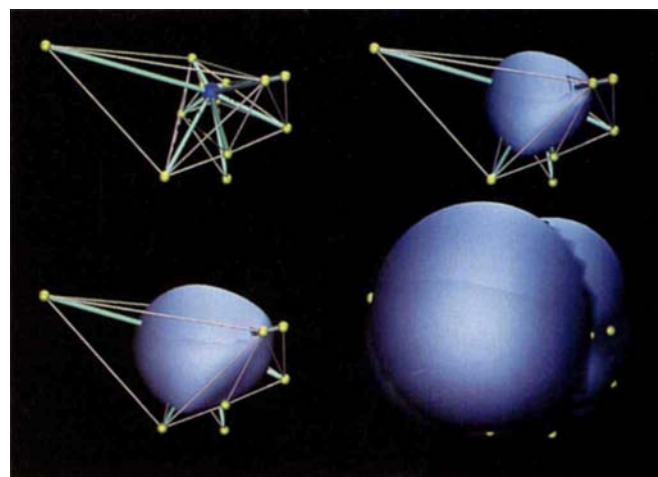
FIG. 4 The isosurface for which the natural-neighbour coordinate is zero for the nodes shown in Fig. 3. The surface is shown from a different perspective than employed in Fig. 3 to highlight the intersection of the (large) spheres. The spheres always intersect at a neighbour node (smallest spheres), and all spheres pass through the central node, which is obscured in this view.

interpolation¹² in which the value of $f(\mathbf{x})$ at a point $\mathbf{x}$ is given by

$$f(\mathbf{x}) = \sum_i N_i(\mathbf{x}) f_i \quad (1)$$

where the weights $N_i(\mathbf{x})$ are the natural-neighbour coordinates of $\mathbf{x}$ with respect to its natural-neighbour nodes, and f_i is the corresponding field value at the node. Natural-neighbour coordinates of the point $\mathbf{x}$ are defined in Fig. 1c. Each natural-neighbour coordinate, $N_i(\mathbf{x})$, can be displayed as an influence function about node i . Examples in two and three dimensions are shown in Figs 2, 3 and 4. They are uniquely defined for any distribution of nodes in any number of dimensions^{5,13}. They also have the important property of being orthogonal, which means that the interpolation will recover the original function values at the nodes, that is, we have:

$$N_i(\mathbf{x}_j) = \delta_{ij} \quad (2)$$



where $\mathbf{x}_j$ is the position of node j (δ_{ij} is the Kronecker delta). Another important property is that they are isoparametric², which means that geometrical coordinates are interpolated exactly, that is, we have:

$$\mathbf{x} = \sum_i N_i(\mathbf{x}) \mathbf{x}_i \quad (3)$$

The third important property is that they are continuously differentiable (C_∞) everywhere except at the nodes, $\mathbf{x}_i$, where all derivatives are discontinuous⁵. These three properties make them very powerful when used as the basis of numerical methods for solving PDEs.

The 'natural-element method'

In this section we present a new approach for solving PDEs which we called the 'natural-element method' (NEM). It is an example of a general Galerkin form of the weighted-residuals method¹⁴ in which the newly defined natural-neighbour coordinates are used as geometrical trial functions. In this new method, the solution $u(\mathbf{x})$ to a general set of PDEs

$$\Lambda(u) = 0 \quad (4)$$

in a domain V , with boundary conditions

$$\Gamma(u) = 0 \quad (5)$$

on boundary S , is approximated by the solution of the following integral equation,

$$\int N_j^T \Lambda \left(\sum_i N_i u_i \right) dV + \int N_j^T \Gamma \left(\sum_i N_i u_i \right) dS = 0 \quad (6)$$

where $u(\mathbf{x})$ has been expanded in terms of trial functions $N_i(\mathbf{x})$ ($i = 1, \dots, n$),

$$u(\mathbf{x}) \approx \sum_i N_i(\mathbf{x} - \mathbf{x}_i) u_i \quad (7)$$

The u_i are the estimated values of the solution, $u(\mathbf{x})$, at the nodes $\mathbf{x}_i$.

In FEM², nodes are usually defined at the vertices of triangular or rectangular elements in two dimensions, or polyhedra in three dimensions, and polynomials are used as trial functions within each element. In this case the trial functions are only continuous to the order of the polynomial across element boundaries. In addition, if the PDE is of high order then the polynomials must also be of high order, which often places constraints on the positions of nodes, for example, nodes at the vertices and faces of triangular or rectangular elements. In NEM, natural-neighbour coordinates are used as trial functions and,

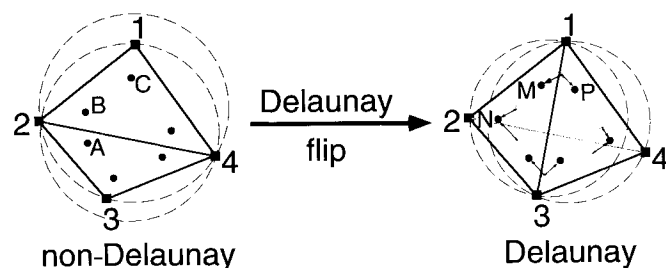


FIG. 5 The basic 'flip operation' used to update the triangulation after each deformation step. Every triangle is checked against each of its neighbours to ensure that the triangulation satisfies the in-circle test: no circle constructed around the corners of the Delaunay triangles contains any node. If two neighbouring triangles do not satisfy the test (note that node 1 is inside the circle built around nodes (2 3 4)) then the side common to the two triangles (2 4) is removed and a new side is added (1 3). The tensors known at the three integration points inside triangles (1 2 4) and (2 3 4) are interpolated onto the integration points inside triangles (1 2 3) and (1 3 4) by simple averaging and splitting, for example $\sigma(\mathbf{N}) = [\sigma(\mathbf{A}) + \sigma(\mathbf{B})]/2$ and $\sigma(\mathbf{M}) = \sigma(\mathbf{P}) = \sigma(\mathbf{C})$.

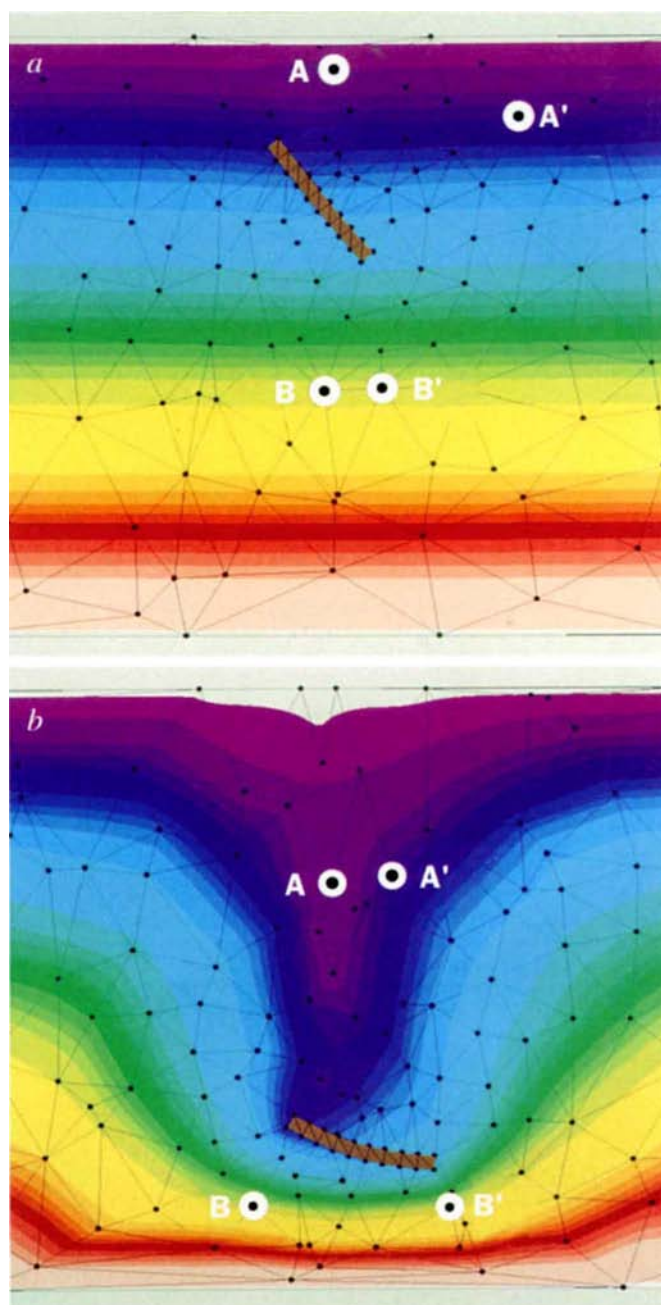


FIG. 6 Natural-element method (NEM) solution of a Stokes flow problem in which motion is driven by the fall of an elasto-plastic plate denser than the viscous fluid. We solve the Stokes equation²⁰ at the integration points in the linear fluid and the equations of force balance²¹ at the integration points located within the elasto-plastic plate, which obeys the Von Mises failure criterion. The solution is shown at times 1 (a) and 1,000 (b). The nodes on the boundary between the plate and the fluid are shared, which ensures no slip between the fluid and the plate. We use an iterative predictor-corrector scheme to maintain stability in the lagrangian grid update performed at each time step from the computed velocity. The fluid viscosity structure, $\eta(z)$, is carried by the nodes. ($\eta(z) = \eta_0$ for $z > 0.75L$ and $\eta(z) = 10\eta_0$ for $z < 0.75L$, where the box has dimensions $L \times W (=10L)$). The colours from purple to white represent the initial depth of fluid particles. The time-dependent solution shows the plate 'falling' down and dragging along the incompressible fluid which causes a return flow pattern to develop. Notice how the mesh accommodates the movement of the lagrangian nodes: points that were originally natural neighbours (for example, B and B') are moved apart by the divergent flow, whereas points that were originally far apart (for example, A and A') eventually become neighbours.

BOX 1 A new method to calculate natural-neighbour coordinates and their derivatives in three or more dimensions

In any number of dimensions, natural-neighbour coordinates are defined as the ratio of the volume of a second-order Voronoi cell to the volume of the first-order Voronoi cell about a point $\mathbf{x}_p$. First- and second-order Voronoi cells are defined in Fig. 1c, for the 2D case. Because the sum of the second-order cells is equal to the first-order cell, to calculate natural-neighbour coordinates in three dimensions we need only determine the volume of each second-order Voronoi cell. Watson¹³ introduced a method to do this for the 2D case. Here we briefly describe a method that can be generalized to any number of dimensions (a fuller treatment is available: see Supplementary Information). Our approach is based on the fact that all second-order Voronoi cells are convex polyhedra (or convex polytopes in higher dimensions). This means that all points inside a second-order Voronoi cell (in dimension n) satisfy a system of linear inequality constraints,

$$A\mathbf{x} \leq \mathbf{b} \quad (9)$$

where the matrix A has $m+1$ rows and n columns, and $m+1$ is the number of boundaries (sides) of the cell. By definition the first bounding plane is the perpendicular bisector of $\mathbf{x}_p$ and one of its neighbours, which we shall call $\mathbf{x}_0$, and the m remaining are the bisectors of $\mathbf{x}_0$ and the nodes which are neighbours of both $\mathbf{x}_p$ and $\mathbf{x}_0$. In two dimensions, these facts can easily be verified from Fig. 1c, and they may be generalized readily to any number of dimensions.

A recursive formula for calculating the volume of any convex polytope in any number of dimensions was given by Lasserre¹⁶,

$$V(n, A, \mathbf{b}) = \frac{1}{n} \sum_{i=0}^m \frac{b_i}{|a_{it}|} V_{it}(n-1, \tilde{A}_{it}, \tilde{\mathbf{b}}_i) \quad (10)$$

where $\tilde{A}_{it}$ is the reduced matrix obtained from A by eliminating the i th variable using the equation $\mathbf{a}_i \cdot \mathbf{x} = b_i$, $\tilde{\mathbf{b}}_i$ is the corresponding reduced vector and a_{it} is the i th element of $\mathbf{a}_i$. The value of t is usually chosen so that $|a_{it}|$ is a maximum. The recursive evaluation of equation (10) is best viewed as a tree structure, with V at the root and the first $m+1$ terms as branches attached to V . In the second level of the recursion, each reduced matrix and vector correspond to a system of inequalities with one less constraint and one less variable. Therefore each branch is attached to m other branches, and so on for each level. After $n-1$ recursions we reach the leaf nodes, and for each of them the system of inequalities is reduced to a set of 1D constraints. In each case the length of the region satisfying all constraints becomes the final contribution to the recursive formula in equation (10).

For any given point $\mathbf{x}_p$ the matrix A and vector $\mathbf{b}$ can be easily determined for each second-order Voronoi cell, and the recursive algorithm leads to an efficient evaluation of their volumes. The accuracy of the second-order Voronoi volumes can be independently verified by comparing their sum to the volume of the first-order cell. We have done this for a test set of 220 nodes in three dimensions; in each case we did not find any discrepancies above the level of machine precision. An example of natural-neighbour interpolation using this formula is given in Figs 3 and 4.

The natural-element method requires derivatives of the natural-neighbour coordinates with respect to the coordinates of the test point $\mathbf{x}_p$. A differentiation of V in equation (10) leads, after some algebra, to a new recursive expression for each derivative, whose computation takes about the same time as the volume itself. The situation is simplified by the fact that only the first row of A and $\mathbf{b}$ depend upon $\mathbf{x}_p$. However, this property is lost for the $i=0$ term in equation (10). It turns out that for these, and all lower branches of the recursive tree, all elements of the reduced matrix $\tilde{A}_{it}$ and vector $\tilde{\mathbf{b}}_i$ become functions of $\mathbf{x}_p$. With the aid of some extensive algebra it is possible to keep track of the dependent terms and calculate all derivatives. The validity of the resulting derivative formulae have been verified by comparison to finite difference estimates for a set of irregularly distributed nodes in up to four dimensions.

therefore, no discontinuities are present across element boundaries as natural-neighbour coordinates are continuously differentiable at all points except at the nodes themselves⁵. Furthermore, like 'classical' trial functions they are orthogonal and isoparametric (equations (2) and (3)), but as they place no constraint

on the positions of nodes they are well suited to solving problems on deforming or self-adapting meshes, even for high-order PDEs like the Navier-Stokes equation.

In the implementation of the natural-element method several computational issues arise. The first, and most important, is the need for a parametric expression for the spatial derivatives of the natural-neighbour coordinates which can be inserted in the integral form of the differential equation (3). In the 2D case, these have been derived by Sambridge *et al.*¹⁵. A new method is outlined in Box 1 which can handle any number of dimensions. An additional advantage with this method is that, unlike with the approach used by Sambridge *et al.*¹⁵, it does not break down when evaluated along the lines (or planes in three dimensions) connecting the mesh nodes. The new approach is based on the formula of Lasserre¹⁶ for calculating the volume of n -dimensional convex polyhedra. An example of a 3D trial function that was computed with this approach is shown in Figs 3 and 4.

The second computational issue concerns evaluation of the integrals in equation (3). In classical FEMs this is usually done using Gauss-Legendre numerical integration over individual elements². This involves estimating the integral as a weighted sum of the interpolated values of the integrand at a set of Gauss-Legendre points inside each element. Often with polynomial trial functions the integrals can be evaluated exactly. However, natural-neighbour coordinates are not simple polynomial expressions and so Gauss-Legendre integration in NEM gives only an approximate integral. For a simple 2D elastic problem, we have evaluated the level of accuracy obtained from a series of 2D integration schemes, namely 1, 3, 4, 7 and 13 integration points per element^{2,17}. In this test problem, three integration points within each Delaunay triangle appeared sufficient. Note, however, that as the integrand need only be evaluated inside, or along the edge of each triangle, the discontinuity in the derivatives of the natural-neighbour coordinates at the nodes is never encountered. Nevertheless the question of finding an optimal integration scheme for natural-neighbour coordinates remains open.

The third issue arises in problems where tensors (such as an initial stress) rather than scalars (such as viscosity or elastic parameters) have to be stored from one time step to the next. 'Tensorial memory' is required in structural mechanics problems for example, whereas 'scalar-only memory' is required in fluid flow or diffusion problems. Unlike scalar quantities, tensors are characterized by orientations (Euler angles or principal directions). Scalar quantities can be evaluated and stored at the nodes of the numerical mesh (which represent material points), whereas tensors must be evaluated at the integration points inside the elements. As the triangulation is updated at each time step, the geometry of the elements changes and a new set of integration points has to be defined, which means that tensors known at the old integration points have to be interpolated onto the new ones. This interpolation will introduce some numerical diffusion which should be minimized by ensuring fine mesh discretization along material interfaces or in regions where tensors (stresses) are discontinuous. It is important to note, however, that in 2D problems, this interpolation is only local. For example, in our 2D implementation of NEM, the mesh update is performed via a series of local Delaunay 'flips' (see Fig. 5) involving two neighbouring elements. During each Delaunay flip, we take advantage of the distribution of the three integration points within the element to devise a simple local interpolation scheme based on two-point averaging and splitting (see Fig. 5). This simple scheme ensures conservation of linear relationships between tensor invariants. A similar procedure is yet to be developed for 3D problems. It is important to stress, however, that this interpolation is needed only in problems involving 'tensorial memory'. No interpolation is required in cases where only scalar quantities need be carried with the deforming mesh, for example in the Navier-Stokes equation, because, unlike tensors, scalars can be stored at the material nodes. Therefore the present

implementation of NEM may be used for these cases in two or three dimensions.

The fourth computational issue concerns the type of method used to solve the set of algebraic equations which results from combining equations (6) and (7),

$$A_{i,j}u_j = b_i \quad (8)$$

In classical FEMS the nodes can be arranged to minimize the bandwidth of the matrix A which ultimately reduces the number of operations needed to solve equation (8). In NEM, the node connectivity is more complex and may change during the calculation, therefore it is not practical to attempt node numbering that will minimize the bandwidth of A . This problem is avoided by using an iterative linear system solver (for example, Gauss-Seidel with over-relaxation¹⁸, or the 'element by element' method with Cholesky factorization¹⁹).

Efficiency of NEM. Sambridge *et al.*¹⁵ have shown that all operations required to compute natural-neighbour coordinates (including the construction of the Delaunay triangulation) are local and, therefore, require a number of arithmetic operations that increases approximately linearly with the number of nodes. This implies that the computational effort required to form the NEM matrix (equation (8)) is similar to the one required in classical FEM. Table 1 shows the results of computations demonstrating that NEM is only about half as efficient as FEM. Furthermore, it must be stressed that the time required to compute the NEM, or FEM, matrix is usually only a small fraction of that needed to solve the resulting algebraic system of equations, and therefore, in practice, there will be little difference in overall computational time between the two approaches.

An example of NEM: fluid-solid interactions

To illustrate the great geometrical flexibility of NEM, we have selected a problem of creeping flow inside a rectangular box driven by a free-falling elasto-plastic plate of greater density than the fluid (Fig. 6a). The Navier-Stokes equations²⁰ with zero Reynolds number are solved in the incompressible fluid whereas the equations of static equilibrium²¹ are solved in the elasto-plastic plate obeying the Von Mises failure criterion²¹. Free slip is assumed on all boundaries. The flow created in the fluid by the falling plate exerts stresses on the plate, which may deform elastically. Increasing internal stresses in the deforming plate leads to irrecoverable plastic deformation (Fig. 6b). This type of coupled fluid-solid system could not be solved using 'classical' eulerian or lagrangian methods. In the eulerian formulation of flow, the boundaries of the solid plate would have to be recomputed at every time step by interpolation onto the 'frozen' mesh, which would eventually lead to inaccuracy due to numerical diffusion during interpolation. In a small-deformation-only lag-

rangian representation, flow in the fluid would require a very large number of remeshing and interpolating steps to avoid excessive mesh deformation.

In our example the initial Delaunay mesh is constructed from a random distribution of nodes with a density inversely proportional to the distance from the plate (Fig. 6a). As expected, the Delaunay triangles are as near equilateral as possible. Nevertheless because the nodal distribution is deliberately non-ideal, some triangles are very elongated. These triangles would be unacceptable in a 'classical' finite element mesh as they would yield inaccurate estimates of derivatives. There is no such restriction in NEM as the derivative estimates at every Gauss-integration point are derived from its surrounding natural neighbours and not just the nodes at the vertices of the triangle containing the point. This means that long thin triangles are less problematical with NEM than in classical lagrangian FEM for two reasons. First, the retention of a Delaunay mesh means that these triangles are less likely to occur, and second, if thin triangles are present the natural-neighbour interpolation ensures that the 'quality' of the derivative estimates everywhere depends on the local node density and not just on the shape of a single triangular element.

In a lagrangian formulation, the position of the nodes is updated at each time step, using the velocity field calculated from the previous time step, which can quickly stretch the mesh and lead to highly distorted elements. This is the reason why lagrangian FEM is usually restricted to small-deformation-only problems. At each time step of NEM we update the nodal positions and connections so that a Delaunay triangulation is maintained. This ensures stability and accuracy of the derivative estimates regardless of how far the nodes have moved. Also we have the added advantage that all fluid properties are carried with the flow without the need for re-interpolation or regridding of the mesh. NEM is therefore very well suited to track material interfaces, deforming boundaries or material properties, even in large-scale-flow problems. In our example, the density of the nodes varies because of the divergent nature of the flow around the plate and along the sides of the box. Because the local nodal density can easily be monitored (using the size of the associated Voronoi cells), it is also straightforward in NEM to dynamically 'inject' new nodes in regions where the density falls beyond some critical value. If this is done, it turns out that only local changes to the Delaunay mesh are required and consequently only those elements of the matrix A that correspond to the new nodes and their neighbours need be recomputed.

Future directions

Our use of the natural-neighbour theory to solve PDEs demonstrates the great geometrical flexibility of the method for solving complex problems on highly irregular evolving grids without compromising accuracy.

The power of NEM is derived from the fundamental geometrical concepts on which it is based, all of which generalize to higher dimensions; for example, triangles in two dimensions become tetrahedra in three dimensions and Voronoi cells become convex polyhedra. More importantly, all natural-neighbour coordinates remain orthogonal, isoparametric and continuously differentiable⁵. The new method we have developed to compute the natural-neighbour coordinates (see Box 1) is valid in any number of dimensions. NEM can therefore be used to solve problems in two or three dimensions. However, because there is, as yet, no direct extension of the 2D 'flip operation' in three dimensions, the Delaunay triangulation has to be computed at every time step by another method (for instance, by using the method of Barber *et al.*⁹). This also means that there is no 3D equivalent of the 2D averaging and splitting strategy that we have used in our implementation of NEM (see Fig. 5), and therefore, in three dimensions, NEM is at present restricted to problems requiring scalar-only memory (like the Navier-Stokes or diffusion equation).

TABLE 1 Relative efficiency of NEM

Number of elements	FEM	NEM		Delaunay triangulation
		Method 1	Method 2	
32	1	1.1	2.1	0.08
200	1	1.2	2.1	0.03
1,800	1	1.36	2.22	0.03

Computing time required to form the NEM matrix normalized by the time taken to form the equivalent FEM matrix for a simple elastic test problem. Quadratic triangular finite elements with three Gauss integration points were used. NEM method 1 is based on the natural-neighbour algorithm used in Sambridge *et al.*¹⁵; NEM method 2 is based on the algorithm described in Box 1. The last column gives the time taken to form the Delaunay triangulation and update it at the end of the time step (normalized by the FEM matrix-formation time). These operations have to be performed in NEM, not FEM, but they do not require substantial computational overhead.

This article has dealt solely with the application of natural-neighbour theory to the weighted residual method for solving PDEs. We expect that many other applications of natural neighbours will appear in the future. For example, it may well be possible to generalize classical finite-difference methods (which rely on estimating derivatives at nodes of a regular grid¹) to irregular meshes. Although the natural-neighbour coordinates

themselves cannot be used to estimate derivatives at nodes, an extension described by Sibson¹² does give first-order derivatives at the nodes. Because most numerical methods are based on the discretization of a field onto a mesh, the theory of natural neighbours raises the exciting possibility of generalizing methods commonly restricted to regular grids to a Delaunay mesh based on any irregular distribution of nodes. □

Received 27 February; accepted 27 July 1995.

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SUPPLEMENTARY INFORMATION. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. We thank H. McQueen, K. Lambeck and D. Watson for stimulating discussions, U. Christensen for his review and D. Whitehouse for producing the 3D ray-traced pictures.

Structure at 2.8 Å resolution of cytochrome c oxidase from *Paracoccus denitrificans*

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The crystal structure at 2.8 Å resolution of the four protein subunits containing cytochrome c oxidase from the soil bacterium *Paracoccus denitrificans*, complexed with an antibody F_v fragment, is described. Subunit I contains 12 membrane-spanning, primarily helical segments and binds haem a and the haem a₃–copper B binuclear centre where molecular oxygen is reduced to water. Two proton transfer pathways, one for protons consumed in water formation and one for ‘proton pumping’, could be identified. Mechanisms for proton pumping are discussed.

Cytochrome c oxidase, a protein complex located in the inner membrane of mitochondria and many bacteria, is the terminal enzyme of most respiratory chains. It catalyses the final electron transfer steps from cytochrome c to molecular oxygen, and is a member of the superfamily of haem–copper containing terminal oxidases (see refs 1–3 for review). In addition to the four protons consumed in water formation per oxygen molecule, up to four protons are electrogenically translocated (‘pumped’) across the membrane leading to a difference between the two sides of the membrane of the electrochemical potential of protons. This difference can be used to drive the synthesis of adenosine 5′-triphosphate from adenosine 5′-diphosphate and inorganic phosphate.

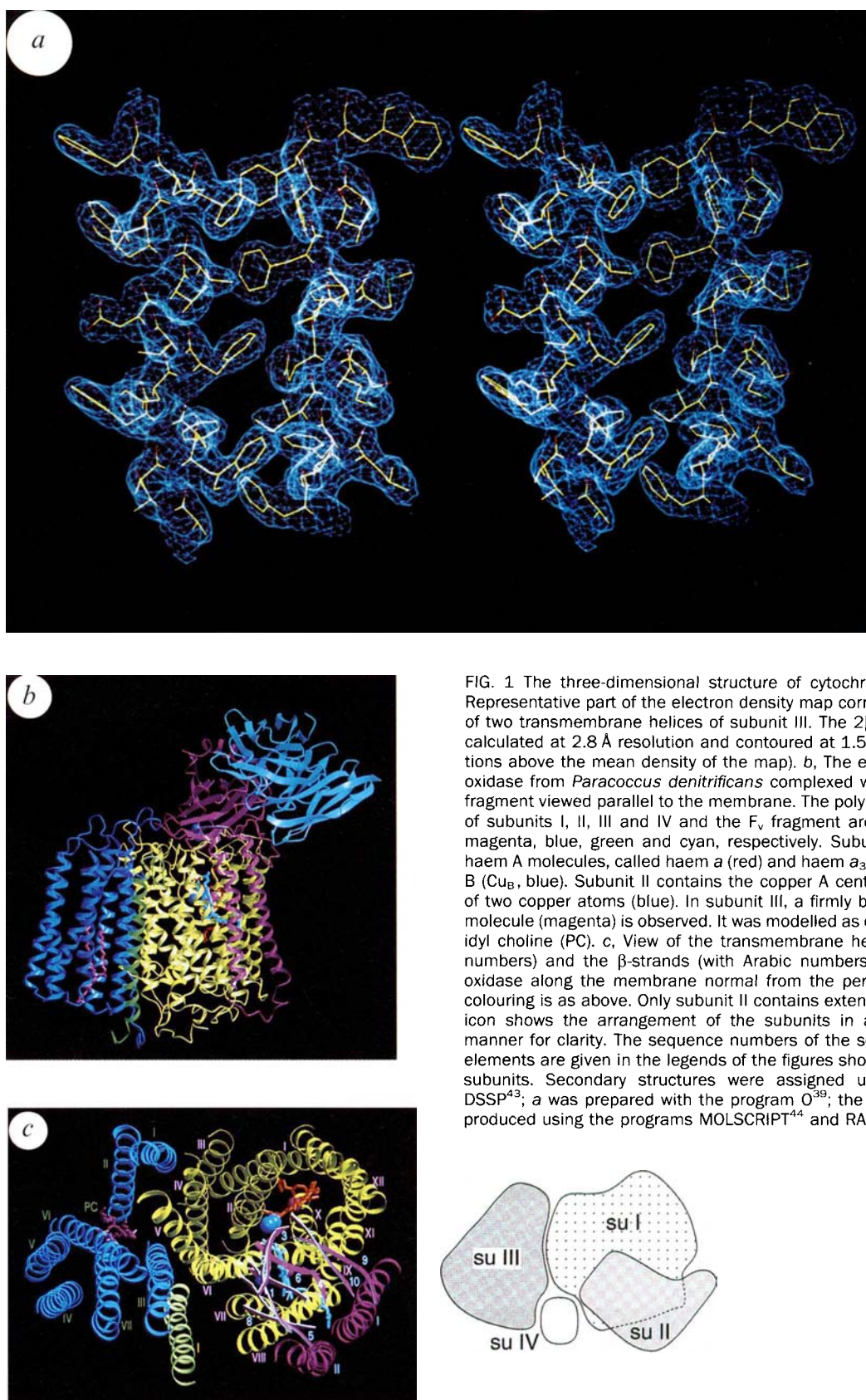
Protein subunit (SU) I of most cytochrome c oxidases contains two haem A molecules, called haem a and haem a₃, and copper B (Cu_B). Haem a₃ and Cu_B form a binuclear centre where molecular oxygen is reduced to water. Electrons from cytochrome c are first transferred to the copper A centre (Cu_A), which is located in protein SU II, and from there to haem a and the binuclear centre. The function of SU III, which is present in all mitochondrial and most bacterial terminal oxidases, is unknown. Up to ten small additional subunits are present in mitochondrial cytochrome c oxidases⁴.

As a prerequisite for understanding the molecular mechanism of oxygen reduction and its coupling to proton pumping, we have determined at 2.8 Å resolution the structure of the four protein subunits containing cytochrome c oxidase⁵ from the soil bacterium *Paracoccus denitrificans* by X-ray crystallography. This became possible by cocrystallizing this membrane protein complex with an F_v fragment of a monoclonal antibody. Here we describe the structure of this cytochrome c oxidase, its protein subunits, the arrangement and mode of binding of the prosthetic groups, and two possible pathways for protons, one for pumped protons and one for protons consumed in water formation. Finally, we discuss mechanisms of proton pumping.

Structure determination

The fully oxidized cytochrome c oxidase from *Paracoccus denitrificans*, complexed with the antibody F_v fragment 7E2, has been crystallized in the presence of 0.1% sodium azide; azide inhibits the enzyme by binding to Cu_B (ref. 6). Data collection, the quality of data and initial phases determined by multiple isomorphous replacement and anomalous scattering are summarized in Table 1. The phases were improved using a series of density modification methods. The refined structural model contains the following 1,329 residues: SU I, 17–554; SU II, 1–252; SU III, 1–273; SU IV, 10–56; V_L chain of the F_v frag-

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ment, 1–108; and VII chain, 1–120. Three copper atoms, two haem A, and one phosphatidyl choline molecule are included in the current model. A representative part of the electron density map for the refined structure at 2.8 Å resolution is shown in Fig. 1*a*.

Architecture of cytochrome c oxidase

A general view of the *Paracoccus denitrificans* cytochrome c oxidase parallel to the membrane is shown in Fig. 1*b*. In projection the part integrated into the membrane has a trapezoidal appearance. The width at the bottom which corresponds to the cyto-

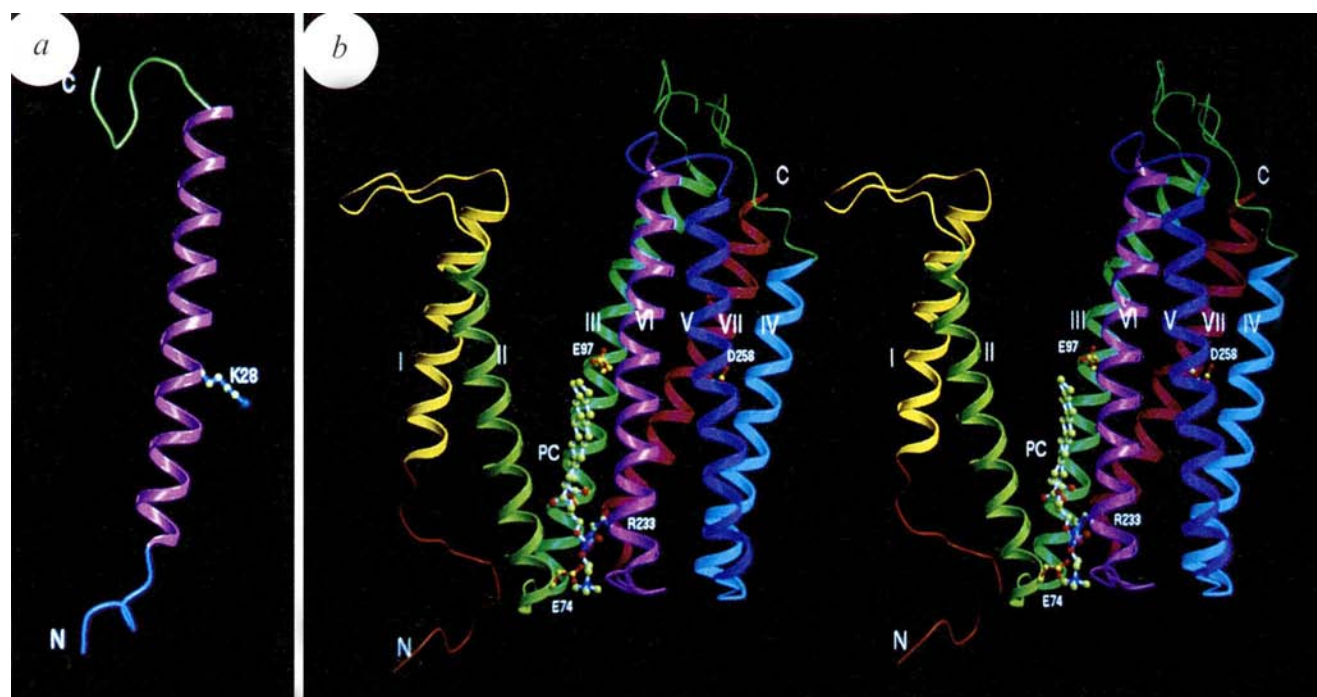


FIG. 2 Structures of subunits III and IV. *a*, View of subunit IV parallel to the membrane. One basic residue, SU IV-Lys 28, is found within the transmembrane helix (SU IV-TM I, residues 16–47). *b*, Stereo view of subunit III as seen parallel to the membrane. The subunit contains 7 transmembrane helices (SU III-TM I, 15–35; II, 48–76; III, 79–114; IV, 139–165; V, 168–196; VI, 203–236 and VII, 244–273). The firmly bound putative phosphatidyl choline molecule is indicated by PC. The

alkyl chains of the lipid are visible up to a length of 12 carbon atoms only. The conserved amino acid residues SU III-Arg 233 and SU III-Glu 74, which form ion pairs with the phosphate and the quaternary ammonium groups of the lipid head group, respectively, and two acidic residues in the transmembrane helices (SU III-Glu 97 and SU III-Asp 258) are also shown.

plasmic surface, is ~ 90 Å; at the top, which represents the periplasmic surface, it is ~ 75 Å. The height of the trapezoid is 55 Å. It comprises 22 membrane-spanning, primarily helical segments and their connections. The globular domain of SU II (see below) is attached to the trapezoid from the top, resulting in a maximum height of 95 Å. The central part of the complex is made up by SU I which binds both haems and Cu_B . Subunit I is associated with SU II at one side and SU III at the other. The amino and carboxy termini of subunit II protrude into the periplasmic space and form a globular domain which contains Cu_A . The antibody fragment, which is also shown in Fig. 1*b*, binds to this globular domain. SU IV consists mainly of one transmembrane helix, but it is nevertheless in contact with the other three subunits (see below). When viewed along the membrane normal, cytochrome *c* oxidase has an oval shape with largest dimensions of 90 and 60 Å (Fig. 1*c*).

Protein subunits and prosthetic groups

Subunit IV. The function of this small subunit is unknown. Its discovery in *Paracoccus* cytochrome *c* oxidase is comparatively recent, and only the sequence of the N-terminal 31 residues has been published⁵. Residues 10–56 are visible in our electron density map. The subunit consists mainly of one transmembrane helix (residues 16–47) with the N terminus on the cytoplasmic side (Fig. 2*a*). As an unexpected feature, one lysine residue is found in the transmembrane helix at position 28. Subunit IV is in contact with all other subunits. The nine C-terminal residues touch residues of three transmembrane (TM) helices (SU III-TM III, SU I-TM VII, SU I-TM VIII). The residue SU II-Arg 198 forms a salt bridge with the C-terminal carboxy group of SU IV. The upper part of the transmembrane helix has indirect contacts with SU III mediated by a presumed phospholipid which is only partially visible in the electron density map and is not included in the model. Recently, the gene coding for SU IV

has been cloned (H. Witt and B.L., unpublished). The available sequences indicate that SU IV is the N-terminal part of a larger (pro) protein of unknown function. However, SU IV is not a typical leader peptide; it may stabilize the *Paracoccus* cytochrome *c* oxidase.

Subunit III. The function of this subunit is also unknown. It does not contribute to the binding of the redox-active prosthetic groups, nor is it involved in proton pumping, as a two-subunit cytochrome *c* oxidase consisting of SU I and II can be isolated⁷, and is active in proton pumping⁸. SU III may be involved in the assembly of the cytochrome *c* oxidase⁹. It possesses seven transmembrane helices that are arranged in an irregular manner. When viewed from the periplasmic space and counted clockwise, the order is I, III, VII, IV, V, VI and II (see Fig. 1*c*). The seven transmembrane helices are divided into two bundles, one formed by the first two helices, and the other by the transmembrane helices III to VII. The two bundles are separated by a large V-shaped cleft (Fig. 2*b*), at the bottom of which one lipid molecule is firmly bound. The electron density is compatible with phosphatidyl choline in a syn-clinal/ β /anti-periplanar conformation (see ref. 10 for definition). The conserved amino acid residues SU III-Arg 233 and SU III-Asp 74 form ion pairs with the phosphate and the quaternary ammonium of the lipid head group, respectively. The alkyl chains of the lipid, which are not visible in full lengths, are lined up in the cleft along helices III and VI. The well-conserved dicyclohexyl carbodiimide binding residue SU III-Glu 97 (ref. 11) is located in the centre of the third transmembrane helix near the cleft. It is therefore probably protonated and forms a hydrogen bond with the conserved SU III-His 219. Its role may be structural.

The cleft of SU III may be a docking site for other membrane proteins. The membrane-anchored cytochrome c_{552} seems to be a physiological electron donor to Cu_A of the *Paracoccus* cytochrome *c* oxidase¹². Its membrane anchor would fit into the cleft,

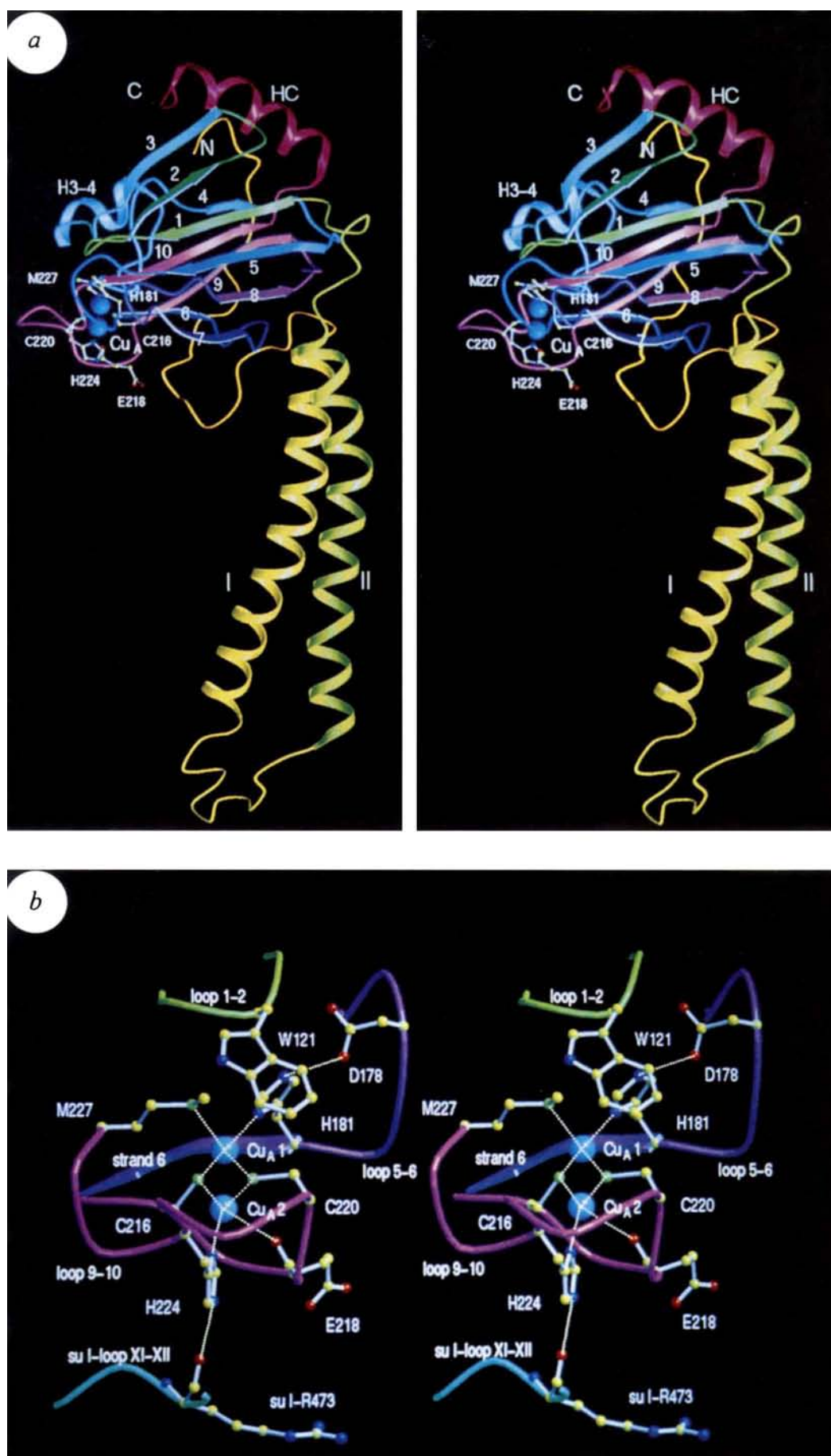
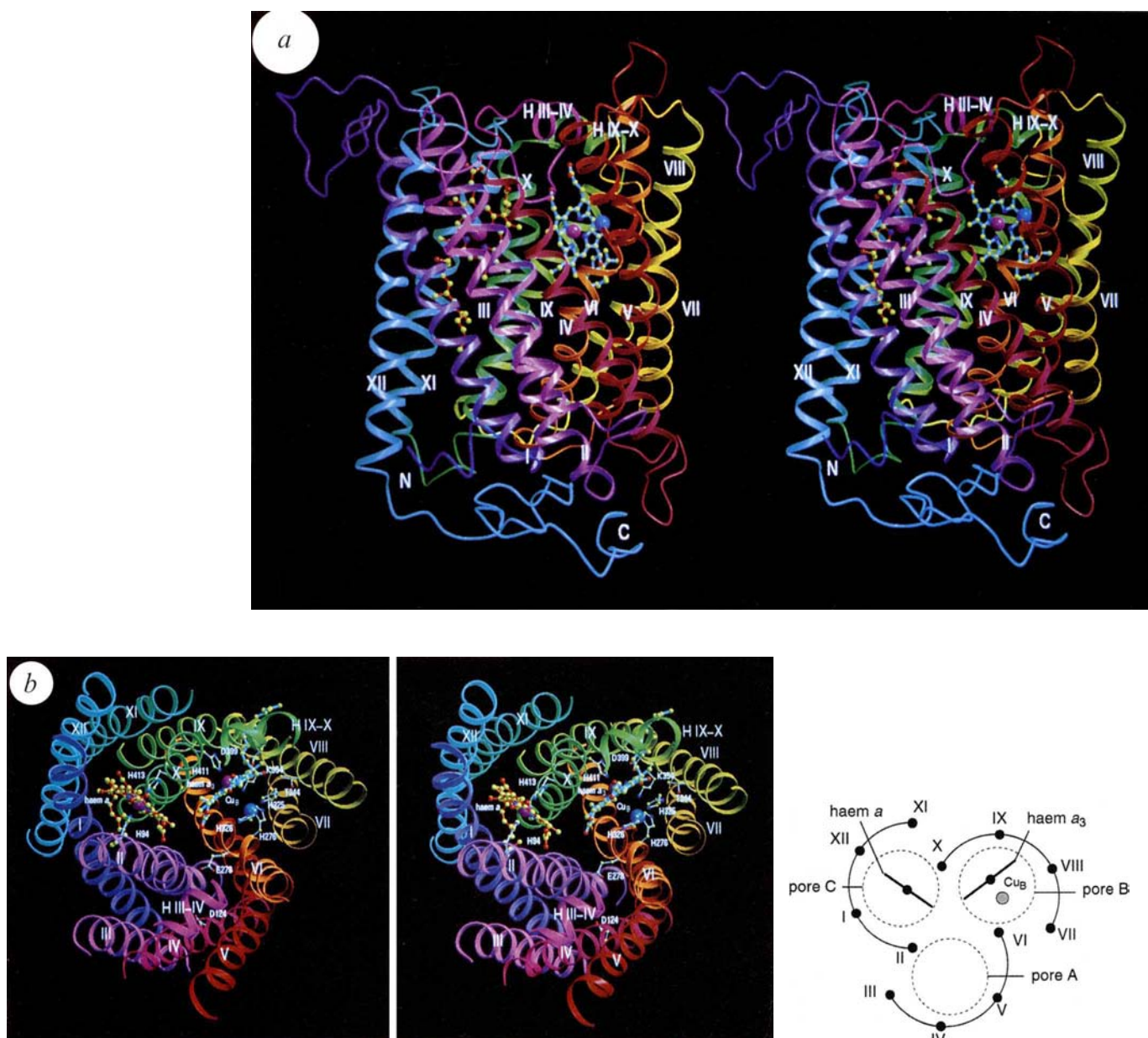


FIG. 3 Structure of subunit II. *a*, Stereo view of subunit II approximately parallel to the membrane. The N-terminal loop (residues 1–26), two transmembrane helices (SU II-TM I, residues 27–59; and II, residues 74–105), including their connection (residues 60–73) and a C-terminal globular domain (residues 106–252) which contains the Cu_A centre are shown. The C-terminal globular domain contains a 10-stranded β -barrel (strand 1, residues 114–119; 2, 123–127; 3, 132–136; 4, 162–164; 5, 169–175; 6, 181–185; 7, 190–194; 8, 200–204; 9, 209–216 and 10, 227–234), and two helices, helix 3–4 (H3–4, residues 140–147) and a C-terminal helix (HC, residues 235–249). The two copper atoms (blue) of the Cu_A centre and their ligands are shown. *b*, Stereo view of the Cu_A centre. The loops providing ligands to the copper atoms are shown and named according to the β -strands which they connect.



and its globular domain might be in an appropriate position to transfer electrons to Cu_A.

Subunit II. Subunit II comprises three segments: an N-terminal loop (residues 1–26), two transmembrane helices, including their connection (SU II-TM I and SU II-TM II, residues 27–105), and a C-terminal globular domain (residues 106–252) which contains Cu_A (Fig. 3a). The N-terminal loop and C-terminal domain interact tightly on the periplasmic side. Both are located above pore B of SU I (see below). The transmembrane helices SU II-TM I and SU II-TM II are in extensive contact with two transmembrane helices of SU I (SU I-TM IX and SU I-TM VIII, respectively; Fig. 1c).

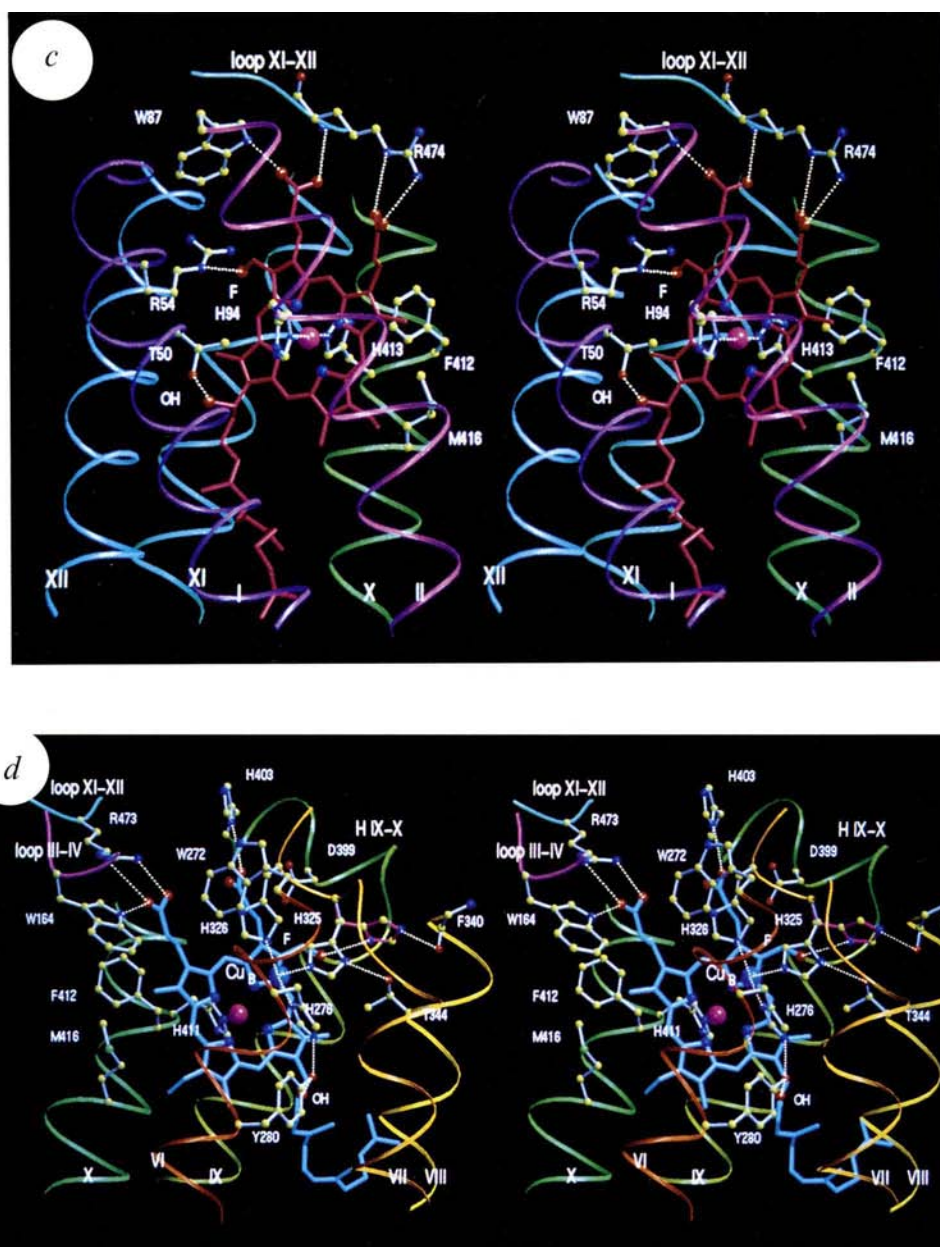
The folding of the C-terminal globular domain containing a ten-stranded β -barrel is similar to that of class I copper proteins such as plastocyanin and azurin, as expected from sequence comparisons^{2,13,14}. The α -carbon atoms of the core region superimpose on plastocyanin¹⁵ with a root mean square difference of 2.2 Å for 74 of the 99 plastocyanin residues. The major difference is the insertion of strands 3 and 4 and the loop between these strands in SU II.

As an important difference to the type I copper proteins, Cu_A has been suggested to be a mixed-valence [Cu(1.5)–Cu(1.5)] complex^{16–20}, which agrees with the crystal structure (Fig. 3b).

The binding site for the two copper atoms (Cu_{A1} and Cu_{A2}) is formed by residues from strand 6 and the loop connecting strands 9 and 10 (Fig. 3b). The ligands of Cu_{A1} are the residues SU II-Cys 216, SU II-Cys 220, SU II-His 181 and SU II-Met 227; those of Cu_{A2} are SU II-Cys 216, SU II-Cys 220, SU II-His 224 and the carbonyl oxygen of SU II-Glu 218 (Fig. 3c). The ligands for each Cu atom form a distorted tetrahedron. The two copper atoms are bridged by two cysteine thiolates. In our model the Cu–Cu distance is 2.6 Å. The copper and the sulphur atoms lie in one plane. The Cu–S_γ (Cys), Cu–S_δ (Met), Cu–N₈₁ (His) and Cu–O (Glu) distances are 2.3, 2.7, 2.1–2.2 and 3.0 Å, respectively. The Cu–S_γ (Cys) distances are difficult to determine because overlap of the electron densities of the copper and sulphur atoms makes it difficult to locate the sulphur atoms precisely. The bond lengths for Cu–S_δ and Cu–O are similar to those observed in type I copper proteins¹³.

The residue SU II-Trp 121, which is within 3.2 Å of SU II-Met 227, might be involved in electron transfer from cytochrome *c* to Cu_A. SU II-Trp 121 and SU II-Asp 178, which can form a hydrogen bond to the Cu–ligand SU II-His 181, shield the Cu_A centre from the solvent. Very close to this site, is the most likely cytochrome *c* binding site, the corner formed by the globular domain of SU II and the flat periplasmic surface of SU I (Fig.

FIG. 4 Structure of subunit I. *a*, Stereo view of subunit I parallel to the membrane. The subunit contains 12 partly interrupted transmembrane helices (SU I-TM I, residues 27–59; II, 84–121; III, 130–151; IV, 178–206; V, 218–251; VI, 263–298; VII, 304–322; VIII, 334–362; IX, 370–395; X, 404–430; XI, 441–468 and XII, 483–513) and two helices parallel to the membrane (helix III–IV, residues 167–174, and helix IX–X, 396–403). Haem *a* (red), haem *a*₃ (cyan) and Cu_B (blue sphere) are also shown. *b*, Stereo view of subunit I along the membrane normal from the periplasmic side. Only the helices are shown together with some important residues. The icon shows schematically the arcs of helices and pores A, B and C formed by them. *c*, Stereo view of haem *a* and surrounding amino acid residues. The symbols OH and F indicate hydroxyl and formyl groups of haem *a*, respectively. *d*, Stereo view of the binuclear centre of haem *a*₃ and Cu_B. The side chain of SU I-His 325 may possess two alternative conformations: in one (white) the side chain forms a bond to Cu_B and a hydrogen bond to SU I-Thr 344, and in the other (red) it forms hydrogen bonds to the formyl group of haem *a*₃ and the carbonyl oxygen of SU I-Phe 340.



1*b*). A part of the loop connecting transmembrane helices III–IV of SU III might also be involved in docking cytochrome *c*. This area contains 10 acidic residues which could interact with lysine residues on the surface of cytochrome *c* (ref. 21).

SU II-His 224 might be involved in electron transfer to haem *a*. The N_{ε2} atom of this histidine could donate a hydrogen to the carbonyl oxygen of SU I-Arg 473 in loop XI–XII of SU I. Several residues of this loop are in contact with the propionate groups of haem *a*. The distance from the conjugated π-electron system of SU II-His 224 to that of haem *a* is 11.9 Å, whereas it is 15.4 Å to that of haem *a*₃. The distances from the lower Cu_A atom to the haem *a* Fe is 19.5, to the haem *a*₃ Fe is 22.1 Å. The shorter distances to haem *a* are in agreement with the observed direct electron transfer from Cu_A to haem *a*.

Subunit I. SU I contains 12 membrane spanning segments, SU I-TM I to SU I-TM XII, with preferentially helical secondary structure (Fig. 4*a*). It shows an unexpected approximate threefold rotational symmetry. When viewed from the periplasmic side, the 12 segments appear to form three symmetry related semicircular arcs with segments II, VI and X being closest to the rotation axis, and segments XI, III and VII most distant

(Fig. 4*b*). The order of the helices is sequential in an anticlockwise manner. Segments XI, XII, I and II form the first semicircle II–VI the second, and VII–X the third. The semicircles together with the last segment of the previous semicircle have a pore-like appearance. In such a description, ‘pores’ A, B and C are formed by transmembrane segments II–VI; VI–X; and X–XII, I and II, respectively. Segments II, VI and X are part of the walls of two pores each. The ‘pores’ are blocked, pore A by mostly conserved aromatic residues, pore B by haem *a*₃ and Cu_B, and pore C by haem *a* and its hydroxyethylfarnesyl side chain (see also Fig. 4*c*). The membrane-spanning segments are connected by 11 loops referred to as SU I-loop I–II to SU I-loop XI–XII. In general, the loops on the periplasmic side are longer (8–26 residues) than those on the cytoplasmic side (5–10 residues). Long periplasmic loops fold back into the subunit so that a flat surface is generated. Only SU I-loops III–IV and IX–X contain short helices which are parallel to the membrane plane. Loop I–II contains a non-conserved disulphide bridge formed by SU I-Cys 66 and SU I-Cys 80. The N and C termini are on the cytoplasmic side. The N-terminal 16 residues are disordered and not visible in the electron density map; the C-terminal 41 residues are of

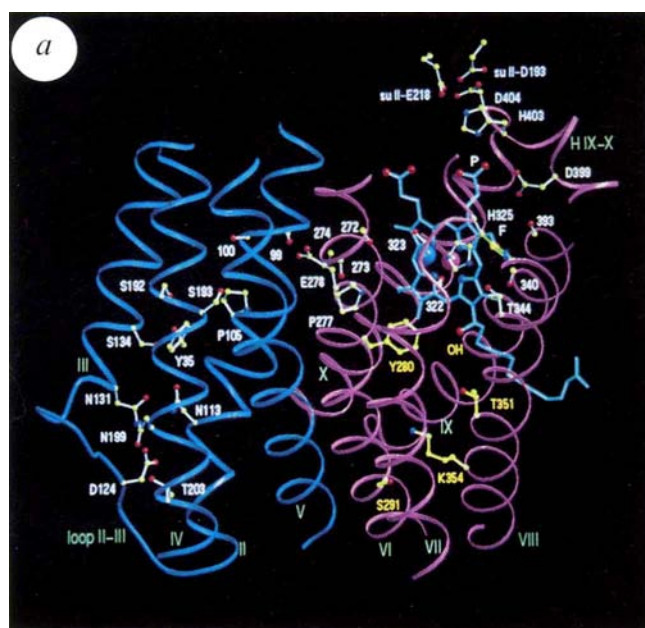
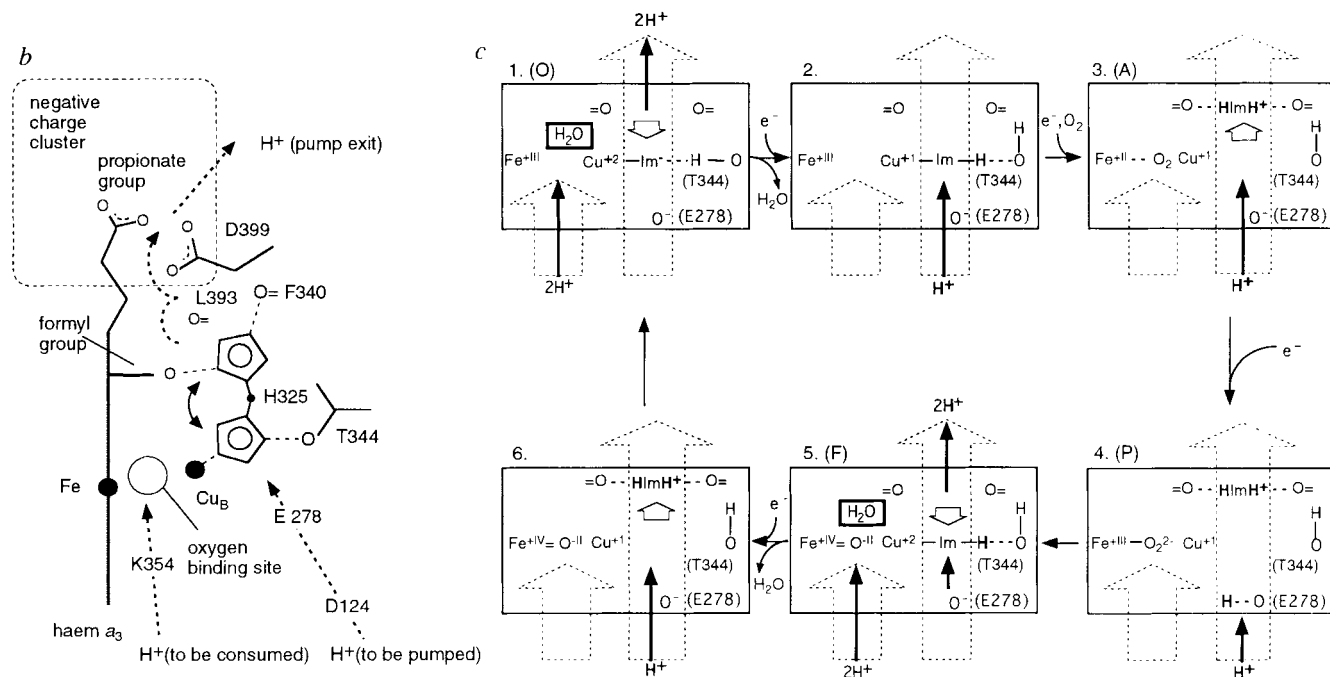


FIG. 5 Proton pathways and possible mechanism of proton pumping. *a*, The proton pathways in subunit I. Residues in the pathway for pumped protons and protons consumed in water formation are labelled in white and yellow, respectively. Two alternative conformations of SU I-His 325 are shown in white and green, respectively. Residues in the negative charge cluster, which is a possible Mn/Mg binding site, are labelled in blue. Important backbone carbonyl groups are labelled with the sequence number only. The symbols OH, F and P indicate hydroxyl, formyl and propionate groups, respectively, of haem a_3 . The segments forming pore A are shown in blue, except for segment SU I-TM VI, those forming pore B in purple. *b*, Schematic representation of the proton pathways and the histidine shuttle. The thick broken lines represent possible proton pathways. *c*, Histidine cycle/shuttle mechanism for coupling of proton pumping to oxygen reduction to water (short version). The numbers above the boxes describe the states referred to in the text. The symbols are as follows: O, fully oxidized; A, the compound A; P, the peroxy; and F, the oxoferryl (F) states^{1,30}. Protons to be pumped enter the boxes at the bottom right and leave at the top along the broken arrows. They are indicated in bold. Protons to be consumed enter from the lower left. Movements of the SU I-His 325 side chain ('Im') are indicated by the short white arrows in the boxes.



extended secondary structure with turns and form the bottom of SU I.

Haem a . Haem a is a low-spin haem with two histidine residues as axial Fe-ligands. It transfers electrons from Cu_A to the binuclear centre. The haems are characterized by non-covalent binding to the protein and the presence of a formyl group at C8 and a hydrophobic hydroxyethylfarnesyl group at C2 (Fig. 4c). The axial Fe-ligands of this haem are His 94 from SU I-TM II and His 413 from SU I-TM X as predicted²². The haem a formyl group accepts a hydrogen bond from the side chain of SU I-Arg 54. The propionate groups of haem a are connected with the Cu_A centre by SU I loop XI-XII. The side chain of SU I-Arg 474 apparently forms a hydrogen bond/ion pair with one of the two propionates, the other propionate is hydrogen bonded to the peptide carbonyl oxygen of SU I-Arg 474 and the side chain of SU I-Trp 87. In contrast to haem a_3 , the hydroxyethylfarnesyl group of haem a remains in pore C. It is tightly sur-

rounded by hydrophobic residues, so access to haem a from the cytoplasmic side is completely blocked.

Haem a_3 and Cu_B . The binuclear centre formed by haem a_3 and Cu_B is the catalytic centre for O_2 reduction. It is located in pore B, but residues of loops III-IV, XI-XII and the horizontal helix in loop IX X also contribute. The haem a_3 Fe appears to be fivefold coordinated with SU I-His 411 from SU I-TM X as an axial protein ligand. The Fe is ~ 0.7 Å out of the haem plane towards the histidine. All the polar groups of the haem seem to form hydrogen bonds or salt bridges (see Fig. 4d for details). The hydroxyethylfarnesyl group leaves pore B and penetrates into the lipid bilayer to allow access of protons from the cytoplasmic space (see below). The Cu_B ion is 5.2 Å away from the haem a_3 iron. Molecular oxygen is supposed to bind between the haem a_3 Fe and Cu_B . The electron density map indicates the presence of a solvent (or azide) molecule there. SU I-His 276 ($\text{N}_{\delta 1}$) and SU I-His 326 ($\text{N}_{\epsilon 2}$) are Cu_B -ligands. The residue

TABLE 1 Statistics for X-ray data collection and phase determination

(a) Diffraction data												
Derivatives	Concentration (mM)	Soaking time (days)	$d_{\min}$	No. of measurements	Unique data	Completeness (%)	R_{merge}^* (%)					
Native			3.0	193,855	63,685	89.9	6.7					
Native (high resolution)			2.8	301,392	76,703	88.9	10.8					
MMA (c-axis)	1.0	3	3.5	102,143	38,506	85.8	6.9					
MMA (a-axis)	1.0	5	3.5	115,572	39,325	87.4	7.9					
HgCl ₂	0.5	3	3.5	113,997	39,943	88.9	7.0					
Thiomersal	1.0	7	3.5	81,668	36,229	80.2	6.6					
Pb(CH ₃) ₃ Cl	5.0	5	3.5	106,586	39,406	87.1	7.9					
Pb(C ₂ H ₅) ₃ Cl	5.0	5	3.5	92,333	37,251	83.3	6.8					
Tl(C ₆ H ₅) ₂ Cl	5.0	7	3.5	98,344	38,699	84.6	8.3					
(b) Phase determination												
Derivatives	R_{deriv} (%)	No. of sites [†]	$R_c^{\ddagger}$	Phasing power at each resolution bin (Å)								
				(10.5)	(8.2)	(6.7)	(5.7)	(4.9)	(4.3)	(3.9)	(3.5)	Total
MMA (c-axis)	14.3	7	0.69	0.72	0.91	1.44	1.61	1.29	1.00	0.96	0.85	1.04
MMA (a-axis)	15.2	7	0.71	0.76	0.86	1.26	1.62	1.25	0.98	0.86	0.76	0.99
HgCl ₂	14.9	6	0.74	0.52	0.85	1.11	1.25	1.21	1.05	0.86	0.70	0.87
Thiomersal	16.3	9	0.71	0.76	0.91	1.49	1.57	1.15	0.88	0.92	0.76	1.00
Pb(CH ₃) ₃ Cl	14.2	5	0.76	0.78	1.00	1.16	1.28	0.95	0.73	0.56	0.52	0.81
Pb(C ₂ H ₅) ₃ Cl	16.2	5	0.73	0.53	0.71	0.81	1.04	0.99	0.76	0.63	0.61	0.73
Tl(C ₆ H ₅) ₂ Cl	16.8	5	0.80	0.52	0.94	1.13	1.07	0.83	0.54	0.46	0.36	0.64
Figure of merit				0.84	0.91	0.89	0.82	0.71	0.56	0.44	0.34	0.58

Crystallization. Cocrystals of the conformation-specific antibody F_v fragment 7E2 and the cytochrome *c* oxidase from *Paracoccus denitrificans* were obtained as described (C.O., S.L., B.L. and H.M., submitted). They belong to space group P4 with $a = b = 206.7$ Å, $c = 83.5$ Å, with one complex in the asymmetric unit. **Diffraction data.** X-ray data collection was carried out at station BL6A2 of the Photon Factory, Japan. Intensity data were obtained using a Weissenberg camera for macromolecular crystallography and imaging plates as detector³². Four crystals were used for native data collection and one for each heavy-atom derivative. For refinement, a high-resolution data set was collected up to 2.8 Å resolution using 8 crystals. Data for mercury derivatives were collected at 1.00 Å, and all other data at 0.94 Å. Data were processed with the HKL programs DENZO and SCALEPACK³³. **Phasing.** Major heavy-atom sites were determined from difference Patterson maps and minor sites and relative positions of sites between derivatives were determined using the difference-Fourier technique. For these purposes a series of programs in the CCP4 suite was used³⁴. MIRAS (multiple isomorphous replacement with anomalous scattering) phasing and refinement of the heavy-atom positions were done using the program MLPHARE³⁵. MIRAS phases were calculated up to 3.5 Å resolution. The overall figure of merit was 0.58. **Density modification.** Because diffraction from the crystals showed a very large overall B-factor and high anisotropy, we had to apply an artificial overall B-factor to the intensity data to obtain an interpretable electron density map. The intensity data were scaled to the data of photosynthetic reaction centre at each resolution shell followed by an overall anisotropic B-factor application. Details of the method will be published elsewhere. The optimal modification was monitored with the free *R*-factor³⁶ obtained in the following density modification step. Density modification and phase extension to 3.0 Å was performed with the program DM³⁷. Solvent flattening and histogram mapping were applied. The initial free *R*-factor was 0.461, but it reduced to 0.255 after 200 cycles. The best result was obtained with a solvent content of 75%. For further density modification, the program SOLOMON was used³⁸. The *R*-factor dropped from 0.246 to 0.166 within 32 iterative steps of the modification. The best result again was obtained with a solvent content of 75%. **Model building and refinement.** The atomic model of the cytochrome *c* oxidase was built using the program O³⁹. From the positions of the mercury binding sites and the sequences it became evident that it is the second gene⁴⁰ of subunit I from *Paracoccus denitrificans* which is expressed. As a model for the 7E2 F_v fragment, the structure refined at 1.28 Å was used⁴¹. Simulated annealing followed by conventional positional refinement using the program X-PLOR⁴² resulted in a structural model with an *R*-factor of 21.1% for the data between 6.0 and 2.8 Å resolution. The free *R*-factor of 5% of the data within the same resolution range was 25.6%. The root mean squares deviations from standard values of bond lengths and angles are 0.010 Å and 1.8°, respectively.

* $R_{\text{merge}} = \sum_i \sum_h |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the i th measurement.

† $R_{\text{deriv}} = \sum ||F_{\text{PH}}| - |F_{\text{P}}|| / \sum |F_{\text{P}}|$, where F_{PH} and F_{P} are protein and heavy-atom derivative structure factors, respectively.

‡ $R_c = \sum ||F_{\text{PH}} - F_{\text{P}}| - F_{\text{H(calc)}}| / \sum |F_{\text{PH}} - F_{\text{P}}|$, F_{PH} and F_{P} are defined above, $F_{\text{H(calc)}}$ is the calculated heavy-atom structure factor. Summation is done using centric reflections only.

SU I-Tyr 280, which can form a hydrogen bond with SU I-His 276, and SU I-Trp 272, whose ring is in van der Waals distance parallel to SU I-His 326, may stabilize the Cu_B site. The most interesting residue near Cu_B is SU I-His 325. There is no electron density for its side chain, indicating disorder or multiple conformations. It can be modelled in at least two conformations (Fig. 4d). In one it forms bonds to Cu_B and the O_{γ1} atom of SU I-Thr 344, whereas in the other it forms hydrogen bonds with the formyl group of haem *a*₃ and the carbonyl oxygen of SU I-Phe 340 and is not a Cu_B ligand.

The planes of both haem groups are perpendicular to the membrane, and their interplanar angle is 108°. The shortest distance between the haem *a* and haem *a*₃ molecules is 4.7 Å, and the centre-to-centre distance is 13.2 Å. There are several possible pathways for electron transfer between the haems: (1) a direct pathway; (2) a pathway using the Fe-ligands SU I-His 413 (haem *a* Fe-ligand) and SU I-His 411 (haem *a*₃ Fe-ligand) by means of the connecting peptide backbone; and (3) one by

means of SU I-Phe 412 and SU I-Met 416 with their side chains in contact to both haems.

Proton pathways

For the function of cytochrome *c* oxidase, protons are required for two different purposes. 'Chemical' protons are consumed upon reduction of O₂ and water formation, and 'pumped' protons are translocated from the cytoplasmic to the periplasmic side of the membrane. Because the mutation SU I Asp 124 → Asn blocks proton pumping but not water formation, two separate pathways were expected^{23,24}. In agreement with these results, two possible proton transfer pathways can be identified.

Pore B below haem *a*₃ (Fig. 5a) seems to be the pathway for consumed protons from the cytoplasmic surface to the oxygen binding site. The entrance is made up of a pit surrounded by SU I loops IV-V and VI-VII, and the C-terminal extension (see Fig. 4a). The residues SU I-Ser 291, SU I-Lys 354, SU I-

Thr 351, the hydroxyl group of haem a_3 and SU I-Tyr 280 sequentially contribute to this pathway. With one exception, these residues are connected by hydrogen bonds directly, or possibly, as indicated by unexplained electron density, by a solvent molecule. SU I-Tyr 280 might donate the proton to the oxygen. The exception is SU I-Lys 354, which does not form hydrogen bonds, either to SU I-Ser 291 or to SU I-Thr 351. Because the environment of this lysine residue is completely hydrophobic, it should be deprotonated. In other possible conformations it could form hydrogen bonds to SU I-Ser 291 or SU I-Thr 351.

The lower half of pore A and presumably the upper part of pore B form the pathway for the pumped protons (Fig. 5a). A gap between SU I-loops II-III and III-IV allows access to the entrance of the pathway. Near the entrance, residues SU I-Asp 124, SU I-Thr 203 and SU I-Asn 199 form a gate. The residues SU I-Asn 113 and SU I-Asn 131 follow. Replacement of one of them by hydrophobic ones blocks proton pumping²⁴. Beyond SU I-Tyr 35 a cavity, primarily lined up with hydrophilic residues such as SU I-Ser 193, is found. Electron density for several solvent molecules in hydrogen-bond distances are visible leading to SU I-Glu 278. Interruption of the regular helical structure of transmembrane segment SU I-TM II by SU I-Pro 105 allows the access of protons to SU I-Glu 278.

Beyond SU I-Glu 278 the proton pathway is less clear. There are two possibilities. First, it might lead into pore B and reach the Cu_B -ligand SU I-His 325. The regular hydrogen-bonding pattern of transmembrane segment SU I-TM VI is interrupted in this area, partly as a result of SU I-Pro 277. The carbonyl oxygen atoms not involved in a helical hydrogen-bonding pattern might play an important role in proton conduction by binding solvent molecules. As mentioned above, SU I-His 325 might possess at least two alternative conformations. A switch between these two conformations might allow conduction of protons to the formyl group of haem a_3 , the carbonyl oxygen of SU I-Leu 393, and SU I-Asp 399. The residue SU I-Asp 399, might be important in accepting protons from SU I-His 325. Alternatively, a direct proton transfer from SU I-Glu 278 to one of the haem a_3 propionates might be possible. Upon conformational changes these side chains could approach one another to hydrogen-bonding distance.

A cleft between SU I and SU II could be the exit pathway for the pumped protons. This cleft is hydrophilic owing to the presence of polar side chains in the SU I-loop VII-VIII, the horizontal helix in the loop IX-X, transmembrane helix II of SU II and SU II-strand 7. Nearby a negative charge cluster consisting of SU I-Asp 399, one propionate group of haem a_3 , SU I-Asp 404, SU II-Asp 193, and SU II-Glu 218 exists. SU I-His 403 is placed in the centre of this cluster. The residues homologous to SU I-His 403 and SU I-Asp 404 have been postulated to be part of a Mn/Mg binding site²⁵. We could not find sufficient electron density for Mn, but weaker densities next to SU I-His 403 might be caused by a Mg atom and/or solvent molecules.

Mechanism of proton pumping

As outlined above, the most likely pathway for the pumped protons includes SU I-His 325 with two possible conformations related to proton conduction. This observation suggests that Cu_B and its ligand SU I-His 325 might play a crucial role in proton pumping. In an attempt to couple the chemistry of oxygen reduction directly to proton pumping of the terminal haem-copper-oxidases, a 'histidine cycle' mechanism has previously been suggested^{26,27}. Here we propose a mechanism in which SU I-His 325 cycles through the imidazolate, imidazole and imidazolium states, and shuttles between two positions (Fig. 5b). This mechanism also obeys the principle of electroneutrality of redox and ligand state changes around the binuclear centre with charge compensations provided only by protonation reactions^{28,29}. Figure 5c shows a short version of our 'histidine cycle/shuttle' mechanism. In state 1, the oxidized or O-state,

SU I-His 325 is in the imidazolate state, owing to positive charges on the Fe and Cu atoms, and bound to Cu_B . Its negative charge is further stabilized by accepting a hydrogen bond from SU I-Thr 344. During single reduction of the binuclear centre a proton is taken up by the pump pathway to maintain electroneutrality, and converts the side chain of SU I-His 325 to the neutral imidazole form. The imidazole ring is now a hydrogen bond donor to SU I-Thr 344 (state 2). During the second reduction another proton is taken up, again by the pump pathway, and will be bound to SU I-His 325. However, this is not possible as long as this residue is a Cu_B -ligand. Therefore it rotates to its alternative binding site, accepting the proton during rotation. Molecular oxygen is bound to the haem a_3 -Fe atom and the so-called compound A is formed (state 3). Upon further reduction the peroxy (P)-state 4 is created (see ref. 1 for review). For charge compensation, a proton has to be taken up. However, because there is no other group with an appropriate pK in the immediate environment available (and accessible from the pump pathway), SU I-Glu 278 is most likely to take up the proton. The chemistry of oxygen reduction then requires the double protonation of the peroxy form, formation of water and creation of the oxoferryl state 5 (F). To keep the overall charge around the binuclear centre constant, the two protons bound to SU I-His 325 have to be released. They are ejected into the exit pathway towards the periplasm. The side chain of SU I-His 325 rotates back. In the imidazolate form it is able to accept the proton from SU I-Glu 278, and it can be bound to Cu_B and SU I-Thr 344 as in state 2. Formation of state 6 occurs analogous to formation of state 3. Two further protons are then taken up by the pathway for protons to be consumed, and water is formed again. The two protons of the SU I-His 325 imidazolium will be ejected by the exit pathway. The side chain of SU I-His 325 swings back and the cycle is now closed.

In the cycle pumped protons are taken up first, and they are ejected by the protons needed for water formation. These two types of protons are provided separately by the two different pathways (Fig. 5b). The delay of the arrival of the protons to be consumed might be explained by the presence of SU I-Lys 354 in their pathway because a conformational change and an energetically unfavourable protonation of this lysine side chain would be necessary for proton transfer.

One objection against this mechanism is that in *bo*-type and *cbb*-type terminal oxidases, the haem does not possess a formyl group to form the hydrogen bond to SU I-His 325. This is not a serious problem, as SU I-His 325 could directly form a hydrogen bond to the carbonyl oxygen of SU I-Leu 393 or to SU I-Asp 399. However, we cannot exclude the possibility that the lack of electron density for the side chain of SU I-His 325 has nothing to do with proton pumping, but is an artefact of the crystallization conditions, especially of the presence of ammonium and azide. It also could be related to the 'resting', 'pulsed', 'slow' and 'fast' states of the enzyme (see, for example, ref. 31). Alternatively, protons might reach the negative charge cluster by the pump channel during the reduction of the binuclear centre and stay there until they are ejected by the exit pathway by long-range electrostatic repulsion by the protons consumed in water formation.

It is evident that further experiments, especially spectroscopic, theoretical and crystallographic studies on wild-type cytochrome *c* oxidase and site-directed mutants, are needed to test the various mechanisms. The cytochrome *c* oxidase from *Paracoccus denitrificans* offers unique possibilities for this purpose. □

Received 19 July; accepted 3 August 1995.

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ACKNOWLEDGEMENTS. We thank the staff of the synchrotron facilities at the Photon Factory, Tsukuba, Japan, and at the ESRF, Grenoble, France for help; J. Eich, C. Münke and in particular H. Müller for technical assistance; and C. R. D. Lancaster for reading the manuscript and discussions. S.I. was supported during part of this work by a grant from the Human Science Frontier Programme, and C.O. by a grant from the Fonds der Chemischen Industrie. Financial support was obtained from the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft (SFB 167) and the Fonds der Chemischen Industrie. H.M. and S.I. are members of the TARA project of Tsukuba University, Japan. The coordinates will be deposited in the Brookhaven Protein Data Bank (PDB).

LETTERS TO NATURE

Slow rotation of the Sun's interior

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THE rotation of the Sun is not that of a rigid body; at its surface, the gas near the poles has a lower angular velocity than that near the equator¹. This latitudinal variation persists to the base of the convection zone, below which the angular velocity becomes approximately uniform^{2,3}. Any variations of angular velocity at much greater depths are, however, poorly constrained^{4–10}. Observations of solar oscillation modes have been used to probe density variations in the Sun; rotational splitting of degenerate modes, although difficult to resolve, provides important constraints on the dynamical structure¹¹. Here we report observations of rotationally split modes made over a three-year period with the Birmingham Solar Oscillations Network. Our results indicate that there is a substantial region inside the Sun that is rotating more slowly than the surface. This situation seems likely to be transient—the minimum-energy state would have all the deeper regions rotating with the same angular velocity—and is at variance with our current ideas about the rotational evolution of main-sequence stars¹². We have no solution to the dynamical problem this poses.

The oscillations that we observe are resonant acoustic waves, called p modes. The eigenfunction of a mode varies in the horizontal approximately as a spherical harmonic Y_l^m of degree l and azimuthal order m , and is characterized also by a principal quantum number n , called the order, which is associated with the radial variation. Eigenfrequencies $\nu_{nl,m}$ would be degenerate with respect to m if the Sun were spherically symmetrical, but rotation shifts the frequencies by a small amount $\delta\nu_{nl,m}$ which

is very nearly an odd function of m , provided that the axis of Y_l^m coincides with the axis of rotation. A magnetic field, or any other symmetry-breaking agent that cannot distinguish east from west, shifts $\nu_{nl,m}$ by an even function of m . Acoustic waves propagate downwards from the reflecting surface layers of the Sun and are refracted back by the rising sound speed. Evidently $\nu_{nl,m}$ depends on conditions only in the region of the Sun within which the waves propagate. The depth of penetration increases as l decreases. The Birmingham Solar Oscillations Network observes the modes of lowest degree, and therefore senses as deeply as it is possible to do with p modes.

Inversions^{2,3,13} of splitting data from modes of intermediate degree have provided a fairly robust view of the Sun's angular velocity $\Omega(r, \theta)$ at radii $r > 0.5 R_\odot$. Broadly speaking, the photospheric variation of Ω with colatitude θ persists through the convection zone, at the base ($r = r_c \approx 0.713 R_\odot$) of which is an abrupt unresolved transition to nearly uniform Ω . The value, Ω_c , of Ω immediately beneath the transition ($\Omega_c/2\pi \approx 440$ nHz) is such that the mean specific angular momentum, averaged over spherical shells, is constant through the transition^{3,14,15} suggesting that there is no net torque exerted across the transition, which is consistent with a steady state. But opinions based on low-degree data about what happens beneath $r = 0.5 R_\odot$ are diverse, some^{4–7} being that rotation is relatively rapid, others^{8–10,13} that $\Omega \approx \Omega_c$. Here we accept the inversions in and immediately beneath the convection zone, and we use our new observations to calibrate a simple model of Ω at greater depths.

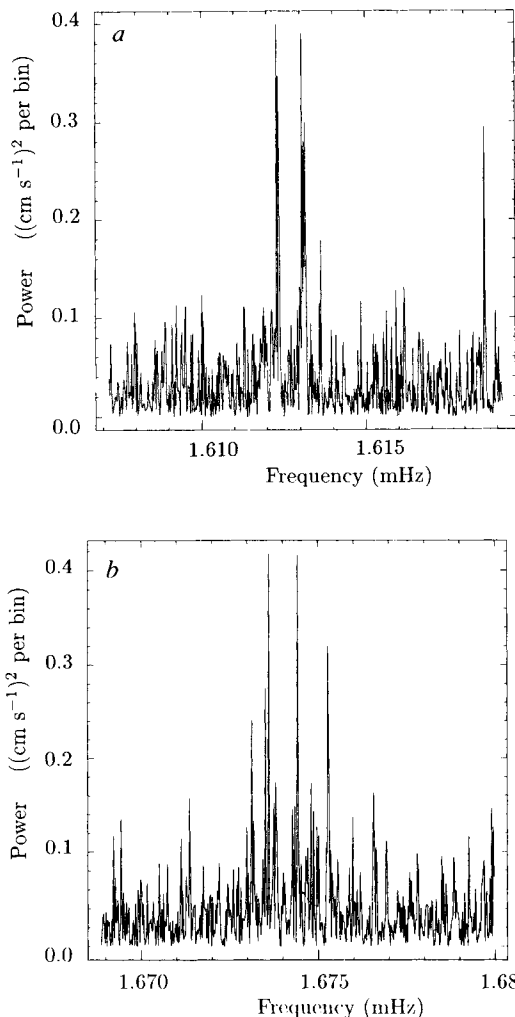
A severe difficulty previously encountered in measuring splitting of low-degree modes arises from the fact that the widths of the lines in the power spectrum are comparable with the splitting; the lines of the components with different m of a (n, l) multiplet are therefore blended, and it is necessary to rely on statistical cancellation of interference effects to infer the splitting indirectly, with risk of biased estimation. We have overcome that difficulty by observing low-frequency modes whose lines are narrow enough for the individual components of a multiplet to be resolved, permitting a direct measurement of the splitting. This has never before been achieved with whole-disk observations.

Observations were made with a network of six resonance-scattering spectrometers located in Tenerife, Chile, California, Eastern Australia, Western Australia and South Africa. With such a global network the confusing effects of daily sidelobes in

the spectra are very much less than they are with single-station data. The instruments, which work with the 770-nm optical resonance transition in potassium, are described elsewhere^{16,17}. We present here analysis of contiguous data from the 32 months from 1 January 1992 to 23 August 1994, split into two sets of 16 months each, having duty cycles of 59% and 74% respectively. The resolution of the spectra is 24 nHz.

The raw data from each station were first calibrated¹⁷, using the known motions of the Earth, to derive velocity residuals. Where necessary, a moving mean over 25 points was subtracted from the residuals to remove low-frequency instrumental effects of up to $\sim 10 \text{ m s}^{-1}$; this process attenuates amplitudes only at frequencies below 1 mHz. The residuals were then combined into time series, using zeros where no data are available. Where data from two or more stations overlap, the data with the highest ratio of power in discrete lines to high-frequency noise were chosen, because these are likely to be the data of the highest quality. The time series were then transformed using standard fast Fourier-transform algorithms to give the power spectra, some of which are illustrated in Fig. 1.

We fitted peaks using a maximum-likelihood technique, assuming that the data/model ratio follows a χ^2 distribution with two degrees of freedom, as expected for the power spectrum of independent stochastically excited oscillators. To determine the mean rotational splitting of each multiplet, we modelled each group of peaks in a (n, l) multiplet as a sequence of equally spaced Lorentzian components of equal width, with relative amplitudes computed from the sensitivity functions of Christensen-Dalsgaard¹⁸. We present in Table 1 the sidereal splittings, and their averages over n for each value of l and each spectrum.

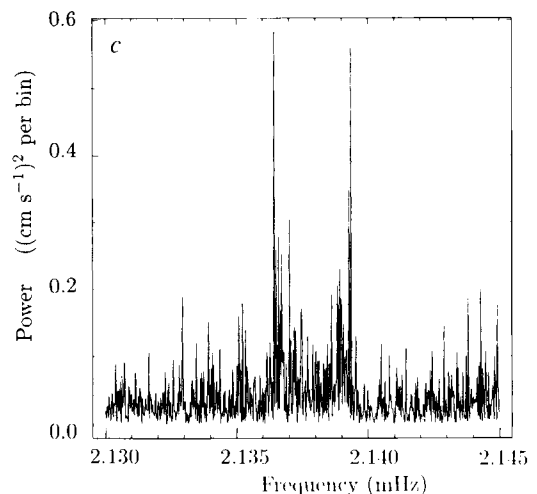


The mean multiplet splittings listed in Table 1 are expected to be close to the sectoral-mode splittings, because the weight in the fitting procedure increases substantially with $|m|$ (see Table 1). They represent averages $\bar{\Omega}/2\pi$ of $\Omega/2\pi$ weighted preferentially near the equatorial plane. The range of n and l over which we have data is insufficient to carry out meaningful inversions to determine the variation of $\bar{\Omega}$ with radius r . Nevertheless, in conjunction with what we know already from the inversions of intermediate-degree modes, we can make some broad deductions about the rotation in the inner regions of the Sun.

The most striking feature of the results presented here is that they correspond to a mean angular velocity less than Ω_c . This suggests that there are regions deep in the radiative interior that are rotating more slowly than the correspondingly averaged equatorial regions of the convection zone. To support that view, we have fitted to the data the splittings associated with a variety of simple models of Ω . In all cases we took $\Omega = \Omega_s$ in the convection zone, where $\Omega_s = \Omega_0 - \Omega_2 \cos^2 \theta$, and Ω_0 and Ω_2 are constants. In the radiative interior, $r < r_c \approx 0.71 R_\odot$, we represented Ω by its appropriately weighted latitudinal average, $\bar{\Omega}$, and ignored any dependence of $\bar{\Omega}$ on l . Then we calibrated the models to fit the frequency splittings listed in Table 1.

We modelled $\bar{\Omega}$ in the radiative interior as a piecewise constant function with up to three segments taking the values $\Omega_c(r < r_c)$, $\Omega_{sh}(r_c < r < r_{sh})$ and $\Omega_e(r_{sh} < r < r_e)$, as illustrated in Fig. 2a. One of the simpler examples has no shell surrounding the core ($r_{sh} = r_c$). In Fig. 2b we depict how the core angular velocity, Ω_c , of that model depends on the radius r_c of the core. The continuous line represents the best fit, the singly hatched region is excluded at 1σ , the cross-hatched region at 3σ .

FIG. 1 Three segments of the power spectrum from dataset C. The two highest peaks in a result from the two visible components of the $l=1$ triplet, of order $n=10$; b is the $l=2$, $n=10$ multiplet and c is $l=3$, $n=13$. As a result of the increase in sensitivity of whole-disk observations with m at fixed l , only the $m=\pm 3$ peaks are obvious in c; they are separated by six times the synodic splitting. Below $\sim 1.7 \text{ mHz}$ the peaks are so narrow that the rotational components are clearly resolved. Above this frequency, the line width increases visibly²⁴: in the range 2.0–2.7 mHz the line width inferred from the Lorentz fitting is comparable with the rotational splitting; above 3 mHz it increases rapidly until it becomes comparable with the spacing between adjacent $l=0$ and $l=2$ modes, and any measurement of the rotational splitting is extremely difficult. Spectra such as those illustrated here are the first that any observation has produced that show low- l modes unambiguously split into well separated components. Below about 3 mHz the amplitudes decline with decreasing frequency and eventually disappear into the noise. The lowest-frequency eigenmodes that we have detected have amplitudes below 1 cm s^{-1} sinewave equivalent.



Although core rotation faster than the surface equatorial value Ω_{sc} (which we take to be 458 nHz) is not excluded at 3σ , it is evidently more likely that the core is rotating (in a mode-weighted average sense) more slowly than the surface.

We are not advocating that the very central regions of the Sun are necessarily rotating more slowly than Ω_{sc} . Indeed, we cannot exclude the possibility of relatively rapid core rotation surrounded by a shell of slowly rotating material, as was deduced by the first rotational splitting inversions¹⁹. However, as is the case with any nonuniform Ω , it behoves us to ask how such an angular momentum distribution could arise, and whether it can be maintained. One possibility is that it is a result of a recent transient advection of angular momentum¹⁴, or is the temporal average of an oscillation about uniform rotation. In either case, the parameters Ω_c , r_c , Ω_{sh} , r_{sh} would be constrained such that

TABLE 1 Sidereal rotational frequency splitting

<i>l</i>	<i>n</i>	ν (μHz)	Splitting (μHz)		
			A	B	C
1	9	1,473	0.430	0.435	0.448
1	10	1,613	0.347	0.441	0.422
1	11	1,749	0.410	0.403	0.408
1	12	1,885	0.480	0.450	0.374
1	13	2,021	0.478	0.446	0.439
1	14	2,157	0.374	0.411	0.335
1	15	2,292		0.368	0.377
1	16	2,426	0.431	0.430	0.499
1	17	2,559			0.430
Mean			0.421 ± 0.020	0.423 ± 0.010	0.415 ± 0.017
2	9	1,536		0.428	0.437
2	10	1,674	0.458	0.455	0.458
2	11	1,810		0.462	0.481
2	12	1,946	0.389	0.397	0.402
2	13	2,082		0.414	0.402
2	14	2,218	0.420	0.409	0.451
2	15	2,352	0.384	0.421	0.434
2	16	2,486	0.448	0.442	0.357
Mean			0.420 ± 0.017	0.428 ± 0.009	0.428 ± 0.015
3	13	2,138		0.472	0.448
3	14	2,274	0.368	0.387	0.391
3	15	2,408	0.437	0.441	0.357
3	16	2,542	0.418	0.421	0.432
3	17	2,676	0.349	0.380	0.405
Mean			0.393 ± 0.024	0.420 ± 0.019	0.407 ± 0.018

The frequencies ν represent the approximate central value $\nu_{n,l,0}$ of the multiplet; the remaining three entries in a row are the rotational splittings inferred from the spectra of the three data sets (A, B and C), converted by dividing the (synodic) frequency spacing between the lorentzians by 2 and adding 31.7 nHz to yield the mean difference between sidereal frequencies of modes whose azimuthal orders m differ by unity. The entries labelled 'mean' at the foot of each set of frequency splittings are uniformly weighted means over principal order n for each degree l , together with the standard errors of those means. No account has been taken of explicit error estimates of the individual splittings. Dataset A extends from 1 January 1992 to 30 April 1993, and dataset C from 1 May 1993 to 23 August 1994. Dataset B, which comprises essentially the last eight months of dataset A and the first eight months of dataset C, from 1 September 1992 to 24 December 1993, has been included because it excludes the earliest data, which are not of such high quality. Before taking the Fourier transform, data acquired before 1 July 1992 with a sampling interval of 42 s were interpolated onto the more recent 40-s sampling interval using sine functions. This kind of interpolation has been found to influence the spectrum only above 8 mHz, and therefore introduces no bias at the frequencies considered here. The amplitudes $A(m)$ of the $l+1$ uniformly spaced lorentzian functions that were fitted to the spectra are independent of the sign of m and have ratios $A(0)/A(2)=0.41$ for $l=2$ and $A(1)/A(3)=0.19$ for $l=3$. The amplitudes of modes with odd $l+m$ are negligible; therefore, the mean frequency separation between the lorentzians fitted to the visible modes represents twice the mean synodic rotational splitting.

the total angular momentum of the core is the same as if the entire solar interior were rotating with Ω_c . However, we have found that under that constraint the value of Ω_c of the best-fitting model never exceeds Ω_{sh} , which cannot readily be explained with simple meridional advection. A more rapidly rotating core is permissible if one admits a 2σ deviation from the mean data; an example is listed in Table 2.

We have also measured the even component of quadrupole frequency splitting. We find $\nu_{n2,0}-0.5(\nu_{n2,-2}+\nu_{n2,2})=+15 \pm 37$ nHz, which differs insignificantly from zero. From this we can impose only an upper bound on the magnetic field. For either a decaying poloidal field of the kind considered by Cowling²⁰ or a toroidal field with a similar distribution of intensity, that bound is ~ 10 MG. A poloidal field would require isorotation on field lines, and possibly even rigid rotation of the radiative interior. However, none of our models with a rigidly rotating radiative zone that match onto the convection zone with continuous mean specific angular momentum, described more fully in Table 2, satisfy the seismic data.

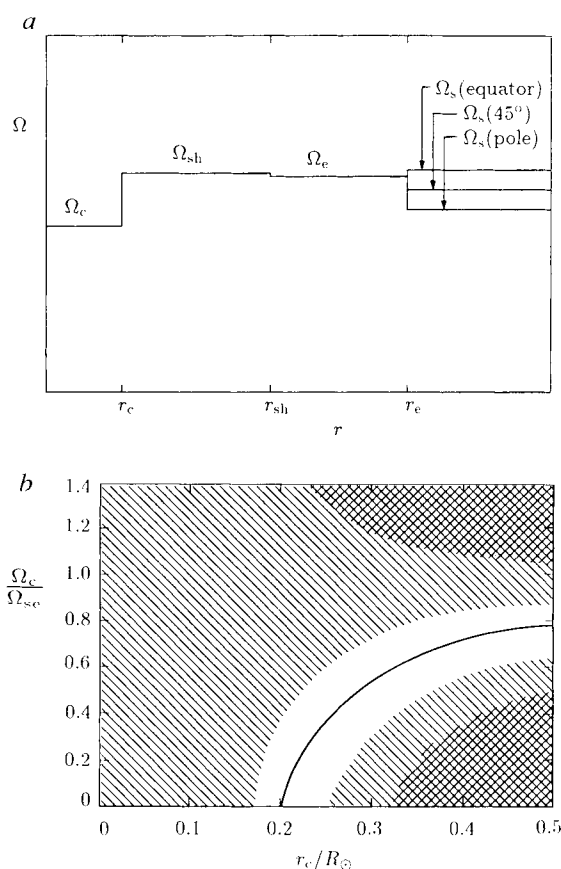


FIG. 2 a, The principal models of angular velocity that have been fitted to the seismic data (Ω is angular velocity, r is radius within the Sun). The radiative interior is typically divided into three regions, a core $r < r_c$, a surrounding shell (if any) $r_c < r < r_{\text{sh}}$, and an envelope $r_{\text{sh}} < r < r_e$, within each of which Ω is independent of r , and is represented by the values indicated in the figure. The convection zone, $r > r_e$, rotates at the surface rate $\Omega_s(\theta)$; values at three colatitudes are illustrated: $\Omega_s(0) = \Omega_0 - \Omega_2$, $\Omega_s(45^\circ)$ and $\Omega_s(90^\circ) = \Omega_0$. It should be realized that we are not necessarily assuming that in the radiative interior Ω is piecewise uniform. The values Ω_c , Ω_{sh} and Ω_e represent mode-weighted latitudinal averages $\bar{\Omega}$, which are most strongly weighted near the equatorial plane—mainly outside the cone defined by $\theta = \theta_0$, where θ_0 is an appropriate average over l of $\sin^{-1} [l/(l+1/2)]$. Except where it is explicitly contradicted, $\Omega_e/2\pi = 0.440 \mu\text{Hz}$. b, Possible values of the core angular velocity Ω_c , in units of the equatorial surface value Ω_{sc} , of a model of the form shown in a with no shell ($r_{\text{sh}} = r_c$). The continuous line represents the best fit to the data, the singly shaded region is excluded at 1σ , and the cross-hatched region is excluded at 3σ .

TABLE 2 Properties of the calibrated models of angular velocity

$r_c/R_\odot$	$r_{sh}/R_\odot$	$\Omega_c/2\pi$	$\Omega_{sh}/2\pi$	Deviation	$J_2(\times 10^{-7})$
0.20	0.20	-0.041		0	1.7
0.25	0.25	0.177		0	1.7
0.20	0.20	0.210		+1 σ	1.7
0.20	0.20	0.724		+3 σ	1.8
0.71	0.71	0.395		0	1.5
0.20	0.45	-0.421	0.576	0	2.4
0.15	0.45	0.600	0.430	+2 σ	1.7
0.71	0.71	0.440		0	1.7

A selection of models of the form illustrated in Fig. 2a, fitted by least squares to the mean constant frequency splitting of 0.416 μHz , augmented, where indicated, by the deviation listed in column 5, where $\sigma = 0.009 \mu\text{Hz}$. J_2 is the implied quadrupole moment of the external gravitational potential, in the usual units of $GM_\odot R_\odot^2$, and was computed under the assumption $\Omega = \bar{\Omega}$ for $r < r_c$; in all cases J_2 is consistent with the radar ranging measurements and General Relativity. $M_\odot$ and $R_\odot$ are respectively the mass and radius of the Sun. (The other variables given as column headings are defined in Fig. 2 legend.) The rotation rate of the convection zone is presumed to be given by $\Omega(r, \theta)/2\pi = \Omega_s(\theta)/2\pi = (0.458 - 0.090 \cos^2 \theta) \mu\text{Hz}$, except in row 5, where it is calibrated to be 0.96 $\Omega_s/2\pi = (0.440 - 0.086 \cos^2 \theta) \mu\text{Hz}$ and matches onto uniform interior rotation $\Omega_c/2\pi = 0.422 \mu\text{Hz}$ with continuous latitudinally averaged specific angular momentum. The motivation for that model was the possibility of there being a large-scale poloidal magnetic field causing the radiative interior to rotate rigidly. We have also considered variants of that model in which the angular velocity in the convection zone is $f(r)\Omega_s(\theta)$, where $f(r)$ increases monotonically from a value f_c at the base of the convection zone $r = r_c$ to unity at the photosphere, the value of f_c being such that the specific angular momentum across the interface with the rigidly rotating radiative interior is continuous. In no case was it possible to find a model that reproduced both the splitting data reported here and the inversions of the data from the Big Bear Solar Observatory (BBSO), which are represented as synodic rotation rates in ref. 18. All the seismic data can be reproduced to within 1 σ by a model with $\Omega = \Omega_s$ in the convection zone and a radiative interior rotating uniformly at 0.395 $\pm 0.025 \mu\text{Hz}$. However, the mean specific angular momentum is no longer continuous at $r = r_c$, suggesting the requirement of a torque. As an example of a different model, one with angular velocity varying linearly in the radiative interior according as $\bar{\Omega} = \Omega_c - (1 - (r/r_c))\Omega_g$ for constant Ω_g satisfies the seismic data with $\Omega_g/2\pi = 116 \pm 57 \text{ nHz}$, with $\Omega_c/2\pi = 116 \pm 57 \text{ nHz}$, implying an equatorial angular velocity rather lower than the values inferred by Goode and Dziembowski²² from the BBSO data in the region $0.4 R_\odot < r < 0.7 R_\odot$, and the recent inferences by Tomczyk *et al.*²³ in $0.2 R_\odot < r < 0.7 R_\odot$. For this adjusted linearly varying model, $J_2 = 1.45 \times 10^{-7}$.

Our splitting data force us to conclude that there is a region of significantly slow rotation in the radiative interior of the Sun. As the minimum-energy state is one of uniform rotation, it is likely that the present situation is either transient or perhaps varies with the solar cycle. That might explain why our present results differ from the relatively high splitting reported earlier in refs 4 and 5. Alternatively, the rotation is steady and is maintained by stresses that result from material motion ultimately driven by thermonuclear energy generated in the core. Whether that motion is slow meridional flow, wave motion or simply turbulence in the convection zone whose influence is transmitted by magnetic stresses into the deep interior is an issue that requires further enquiry. $\square$

Received 29 March; accepted 27 July 1995.

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SUPPLEMENTARY INFORMATION. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. We thank all members, past and present, of the solar groups at Birmingham and the Instituto de Astrofísica de Canarias for their assistance; the staff at the South African Astronomical Observatory (SAAO), the Carnegie Institution of Washington, the Australia Telescope, CSIRO, Australia, and E. J. Rhodes Jr for their hospitality at our Sutherland, Las Campanas, Narrabri and Mount Wilson sites, respectively; P. Fourie for technical help; T. Sekii for assistance with computations; and R. S. Stobie for the facilities of SAAO. We thank PPARC for support.

Improving enzyme–electrode contacts by redox modification of cofactors

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EFFICIENT electron transfer of redox proteins to and from their environment is essential for the use of such proteins in biotechnological applications such as amperometric biosensors and photo-synthetic biocatalysts^{1–3}. But most redox enzymes lack pathways that can transport an electron from their embedded redox site to an electrode^{4,5} or a diffusing photoexcited species⁶. Electrical communication between redox proteins and electrode surfaces has been improved by aligning proteins on chemically modified electrodes^{7–9}, by attaching electron-transporting groups^{10,11} and by immobilizing proteins in polymer matrices tethered by redox groups^{12–14}. Generally these methods involve contacting the enzymes at random with electron relay units. Here we report an approach that allows site-specific positioning of electron-mediating units in redox proteins. We strip glucose oxidase of its flavin adenine dinucleotide (FAD) cofactors, modify the latter with redox-active ferrocene-containing groups, and then reconstitute the apoprotein with these modified cofactors. In this way, electrical contact between an electrode and the resulting enzyme in solution is greatly enhanced in a controlled and reproducible way.

The apoprotein originating from glucose oxidase (from *Aspergillus niger*, EC 1.1.3.4), was prepared by acidification of an enzyme solution to pH 1.7, followed by separation on a Sephadex G-25 column and further purification with charcoal-dextran¹⁵. N⁶-(2-aminoethyl)-FAD (compound 1; Fig. 1)¹⁶ was reacted with N-(2-methylferrocene)caproic acid, (2)¹⁷, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC, to yield the ferrocene-modified FAD analogue N⁶-[N-(2-methylferrocene)-caproylamidoethyl]-FAD (3). Glucose

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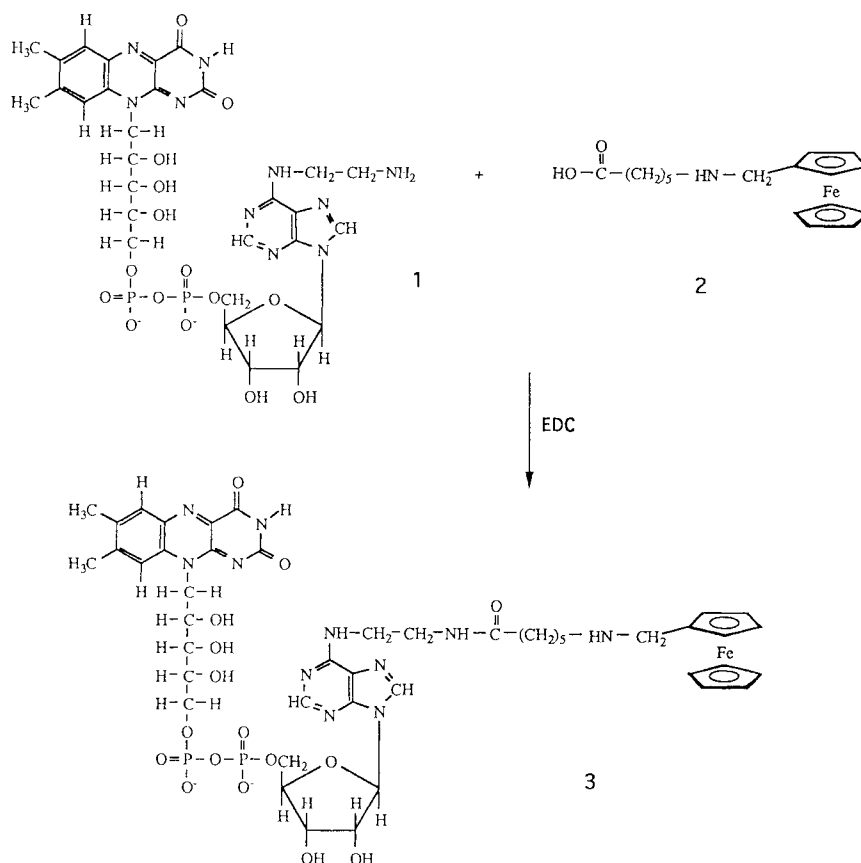


FIG. 1 Structures of compounds **1**, **2** and **3**. EDC is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

oxidase apoprotein was reacted with **3** (in the molar ratio 1:10) to generate the ferrocene-FAD reconstituted glucose oxidase. The loading of the reconstituted enzyme was determined spectroscopically as one molecule of **3** per enzyme subunit. Figure 2 shows the cyclic voltammogram of **3** and of the ferrocene-modified-FAD reconstituted glucose oxidase. The ferrocene-FAD analogue (**3**) exhibits, in an aqueous buffer solution at pH 7.3, two characteristic reversible waves at -0.50 and 0.35 V (with respect to the saturated calomel electrode, SCE). These waves correspond to the two-electron redox process of FAD and the one-electron redox reaction of the ferrocene, respectively. The cyclic voltammogram of **3**-reconstituted glucose oxidase shows only the reversible redox process of the ferrocene unit, implying that the ferrocene component communicates with the electrode where the enzyme-embedded FAD component lacks direct electrical communication with the electrode. Enzymatic assay of the original glucose oxidase apoprotein and the **3**-reconstituted glucose oxidase—using the standard procedure of following the H_2O_2 generated by oxidation of glucose using peroxidase and dianisidine as indicator—revealed that the apoenzyme lacks any biocatalytic activity, whereas the reconstituted enzyme has $\sim 60\%$ of the native glucose oxidase activity.

Figure 3a shows the electrocatalytic anodic currents developed by the **3**-reconstituted glucose oxidase in the presence of different concentrations of added glucose. A gold foil working electrode (area, 0.4 cm^2 ; roughness factor, 1.2), precoated with cystamine monolayer, was employed to prevent non-specific, denaturing, enzyme adsorption on the metal surface. The electrocatalytic anodic current is enhanced as the glucose concentration is increased, and reaches a saturation value. The calibration curve, showing the anodic current at different glucose concentrations, is given in Fig. 3b. The electrobiocatalysed oxidation of glucose can be analysed in terms of the Michaelis-Menten model, Fig. 3c, giving $I_{\text{max}} = 4\text{ }\mu\text{A}$ and $K_m = 2.9\text{ mM}$, where I_{max} is the saturation current and K_m is the Michaelis-Menten constant. Taking into account the surface area and roughness factor of the work-

ing electrode, this maximum current density corresponds to $i_{\text{max}} = 8.3\text{ }\mu\text{A cm}^{-2}$. For comparison, native glucose oxidase, under comparable conditions in the presence of ferrocene carboxylic acid, yields the values $K_m = 3.3\text{ mM}$ and $i_{\text{max}} = 6.3\text{ }\mu\text{A cm}^{-2}$. We conclude that reconstitution of glucose oxidase with the ferrocene-modified-FAD cofactor (**3**) yields a semi-synthetic electroenzyme exhibiting electrical communication between the electrode and the biocatalyst active site.

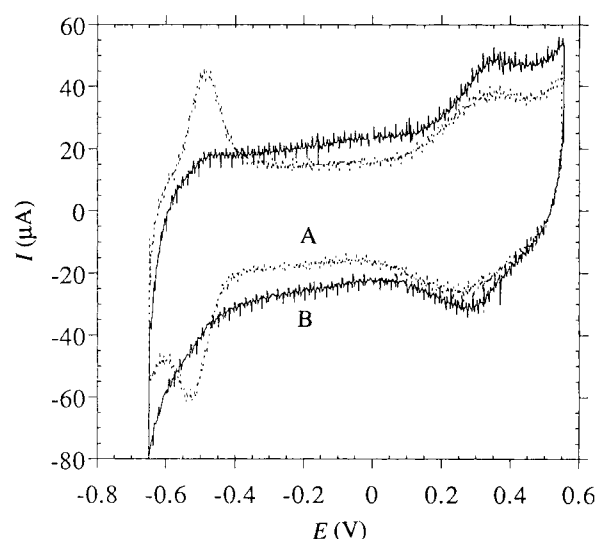


FIG. 2 Cyclic voltammograms. Trace A, ferrocene-modified-FAD analogue (**3**) adsorbed onto a gold working electrode from a $1 \times 10^{-5}\text{ M}$ stock solution. Trace B, **3**-reconstituted glucose oxidase, in solution (1.75 mg ml^{-1}) using a cystamine monolayer-modified Au⁻ electrode. Electrolyte solution; 0.1 M phosphate buffer, pH 7.3. Voltages measured with respect to SCE; scan rate, 1.5 V s^{-1} .

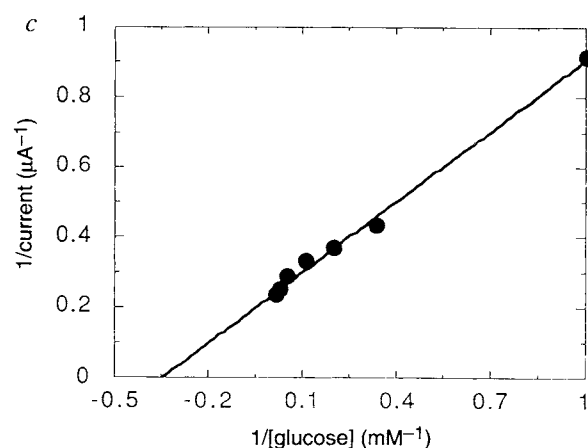
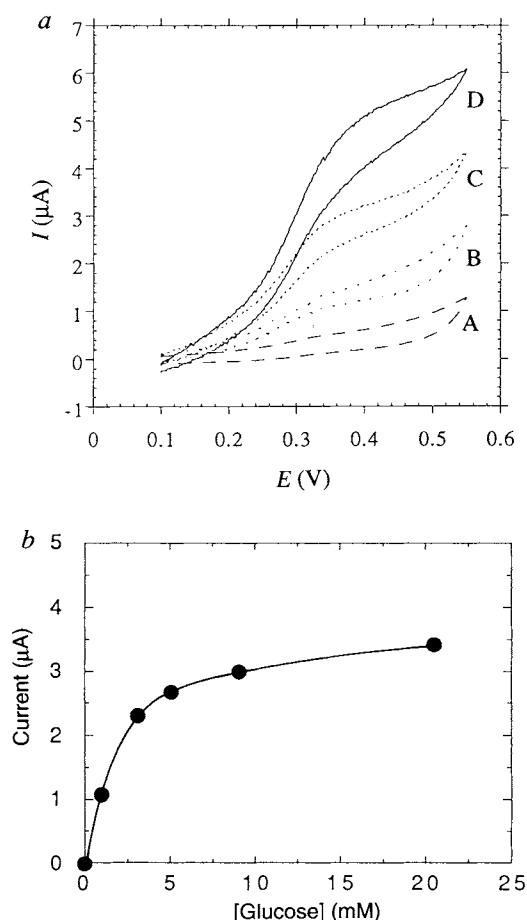


FIG. 3 a, Cyclic voltammograms of systems that contain **3**-reconstituted glucose oxidase, 1.75 mg ml^{-1} , and different concentrations of glucose: trace A, 0 mM; B, 1 mM; C, 3 mM; D, 20.5 mM. All experiments were performed in 0.1 M phosphate buffer, pH 7.3, at $35 \pm 0.5^\circ\text{C}$, using a gold working electrode. Voltages measured with respect to SCE; scan rate, 2 mV s^{-1} . b, Calibration curve of electrocatalytic anodic currents at different glucose concentrations in the presence of **3**-reconstituted glucose oxidase. c, Lineweaver-Burk plot of the electrocatalytic oxidation of glucose by **3**-reconstituted glucose oxidase.

Several control experiments were performed to support the direct electrical communication between the reconstituted enzyme and the electrode. If compound **3** dissolved in an aqueous buffer solution at pH 7.3 undergoes a similar procedure to that employed in the reconstitution—involving filtration through a cut-off filter followed by dialysis—a solution is generated that lacks any electrochemical response by itself or in the presence of native glucose oxidase and glucose as substrate. Also, the **3**-reconstituted enzyme revealed, after chromatographic elution through Sephadex-G25, a similar electrocatalytic activity as observed after reconstitution. Thus any diffusional electrical communication of the reconstituted enzyme through an impurity of **3** is eliminated. Therefore, **3**-reconstituted glucose oxidase represents an organized enzyme assembly exhibiting effective electrical communication with the electrode surface.

The apoprotein derived from D-aminoacid oxidase (DAAO; from pig kidney, EC 1.4.3.3) was prepared¹⁸ as follows. The enzyme was dialysed against a 0.1 M pyrophosphate buffer solution (pH 8.5, containing 1 M KBr and 3×10^{-3} M EDTA), followed by dialysis against a 0.1 M pyrophosphate buffer (pH 8.5). The resulting DAAO apoprotein was reconstituted with **3** by mixing the protein and **3** at a 1:5 molar ratio in a 0.1 M pyrophosphate buffer solution (pH 8.5), followed by filtration through a protein cut-off filter and dialysis against the buffer.

The loading of **3** in the reconstituted DAAO corresponds to one ferrocene-FAD analogue per enzyme. Enzymatic assay showed that the reconstituted enzyme exhibits 20% of the native DAAO activity. (The assay involved the analysis of H_2O_2 formed on biocatalysed oxidation of D-alanine by molecular oxygen, using peroxidase and dianisidine as indicator.) The reconstituted DAAO showed electrical communication with electrode surfaces, and electrocatalytic anodic currents were observed in the presence of D-alanine as substrate. Figure 4 shows the cyclic voltammograms observed on addition of different concentrations of D-alanine to a pH 8.5 electrolyte solution that contains

3-reconstituted DAAO. The electrocatalytic anodic current is enhanced as the concentration of D-alanine increases. The respective calibration curve was similarly analysed in terms of the Michaelis-Menten model, and the values $I_{\text{max}} = 1.9 \text{ μA}$ (or $i_{\text{max}} = 3.96 \text{ μA cm}^{-2}$) and $K_m = 2 \text{ mM}$ were derived for the semi-synthetic electroactive DAAO. Similar to the reconstituted glucose oxidase system, all of the control experiments confirm direct electrical communication between the electrode and the ferrocene-modified-FAD reconstituted DAAO.

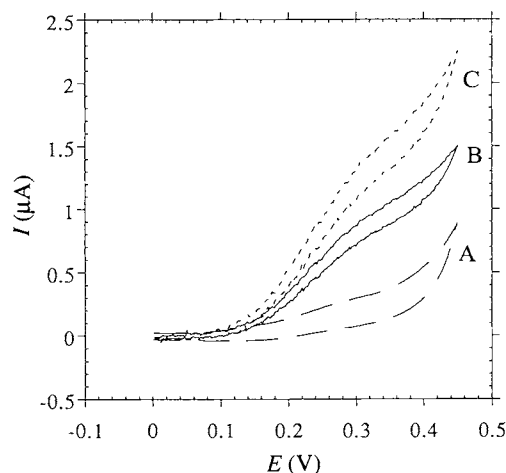


FIG. 4 Cyclic voltammograms of **3**-reconstituted DAAO, 0.38 mg ml^{-1} in the presence of D-alanine: trace A, 0 mM; B, 2 mM; C, 9 mM. Experiment recorded in 0.1 M pyrophosphate buffer, pH 8.5, $25 \pm 0.5^\circ\text{C}$, using a 0.4-cm^2 gold working electrode, roughness factor 1.2. Voltages measured with respect to SCE; scan rate, 2 mV s^{-1} .

We thus conclude that reconstitution of flavoenzymes by a synthetic ferrocene-tethered FAD cofactor provides a means to generate a new class of electroactive biocatalysts for possible use in amperometric biosensors. The structurally defined adducts formed on reconstitution will be suitable for the elucidation of structure-function relations with respect to electrocatalytic activities of flavoenzymes. □

Received 5 May; accepted 27 July 1995.

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ACKNOWLEDGEMENTS. This work was supported by the Ministry of Science of Technology, Israel, and the Commission of the European Communities.

Forest-killing diffuse CO₂ emission at Mammoth Mountain as a sign of magmatic unrest

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MAMMOTH Mountain, in the western United States, is a large dacitic volcano with a long history of volcanism that began 200 kyr ago¹ and produced phreatic eruptions as recently as 500 ± 200 yr BP (ref. 2). Seismicity, ground deformation and changes in fumarole gas composition suggested an episode of shallow dyke intrusion in 1989–90 (refs 3, 4). Areas of dying forest and incidents of near asphyxia in confined spaces, first reported in 1990, prompted us to search for diffuse flank emissions of magmatic CO₂, as have been described at Mount Etna⁵ and Vulcano⁶. Here we report the results of a soil-gas survey, begun in 1994, that revealed CO₂ concentrations of 30–96% in a 30-hectare region of killed trees, from which we estimate a total CO₂ flux of ≥1,200 tonnes per day. The forest die-off is the most conspicuous surface manifestation of magmatic processes at Mammoth Mountain, which hosts only weak fumarolic vents and no summit activity. Although the onset of tree kill coincided with the episode of shallow dyke intrusion, the magnitude and duration of the CO₂ flux indicates that a larger, deeper magma source and/or a large reservoir of high-pressure gas is being tapped.

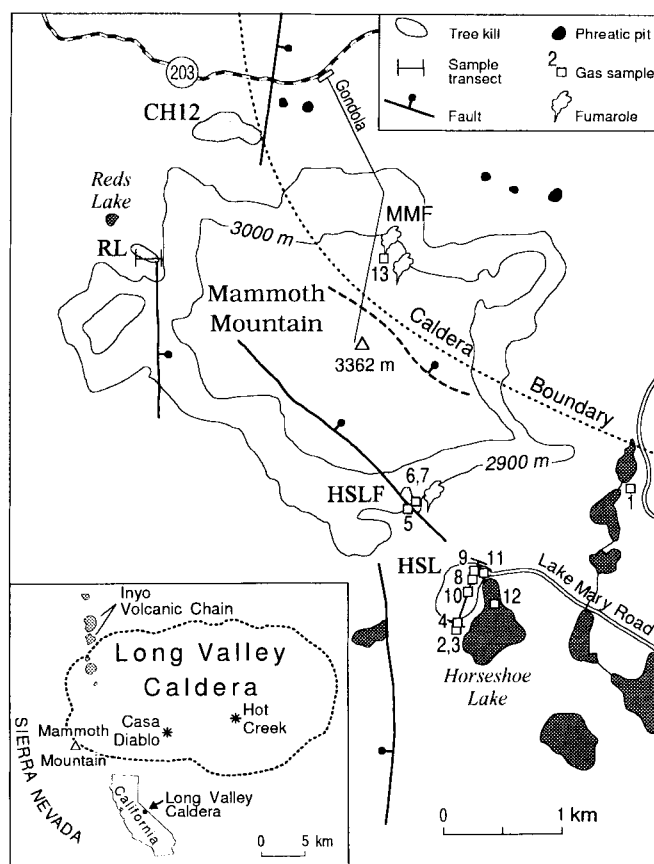


FIG. 1 Maps of Mammoth Mountain and Long Valley caldera showing four areas (light shading) of anomalous tree-kill (labelled HSL, HSLF, RL and CH12), including two transects (RL and HSL) along which [CO₂] has been measured at 0.6 m depth (Fig. 2). Analyses of gas sampled at numbered sites are given in Table 1. Tree line generally follows 3,000 m topographic contour. Also shown are three weak thermal areas with fumaroles at or below boiling temperatures, including the MMF vent that showed increased ³He/⁴He values beginning in 1989 (ref. 3). Inset, map of the 760-kyr-old Long Valley caldera showing Mammoth Mountain at the southern terminus of the Inyo Craters volcanic chain, a north-trending set of domes and phreatic explosion craters dated at 40 kyr to 550 yr BP (refs 1, 2).

Mammoth Mountain lies on the southwestern rim of the 760-kyr-old Long Valley caldera in eastern California (Fig. 1). Areas of tree kill on the flanks of Mammoth Mountain were initially considered to be an effect of four years of persistent drought. But it was later noted that all trees, regardless of age or species, were equally affected within dead or dying areas which gradually increased in number and size. Forest Service biologists eventually determined that biological pests were not the cause of the tree kill (F. Richter, personal communication). In July 1994, we made reconnaissance soil-gas surveys in three tree-kill areas and found that samples from 15 cm depth contained >20% CO₂.

In September and October 1994, we collected ~100 soil-gas samples at 30–60 cm depths within tree-kill areas and from control sites in healthy forest. Carbon dioxide concentrations ([CO₂], vol.%) analysed by a portable gas chromatograph ranged from <1% in healthy forest to >90% at several locations within tree-kill areas. Where [CO₂] exceeded 30%, most trees were dead (Fig. 2). Other lethal agents are not apparent. Results from analyses of a subset of soil-gas samples are summarized in Table 1. Sulphur gas concentrations were indistinguishable from background. High temperatures (to 90 °C) and acidic conditions (but without CO₂) have become prevalent in the soil around a geothermal power plant at Casa Diablo (Fig. 1), where ~20 ha of trees have been killed. In the Mammoth Mountain tree-kill

TABLE 1 Gas characteristics at selected sites

Sample number	Site*	Measured† CO ₂ (vol%)	$\delta^{13}\text{C}^\ddagger$ (‰ PDB)	Measured† O ₂ (vol%)	Measured† N ₂ (vol%)	Oxygen depletion§	CO ₂ (%) Biogenic	¹⁴ C ¶	³ He/ ⁴ He (R/R _A)*	He/CO ₂ (×10 ⁶)
Live trees										
1	HSL (1,500)	0.682	-19.9	20.248	78.082	0.699	102.5	98.2	ND	880
2	HSL (130)	0.515	-14.0	20.562	77.929	0.344	66.8	71.6	ND	1,360
3	HSL (110)	8.438	-3.0	18.496	72.152	0.860	10.2	9.6	ND	83
4	HSL (100)	0.308	-9.2	20.840	77.914	0.062	20.1	ND	ND	1,950
5	HSLF (10)	1.924	-4.8	20.457	76.589	0.090	4.7	ND	ND	364
Dead trees										
6	HSLF	78.530	-4.7	4.318	16.947	0.228	0.3	0.1	5.1(0.4)	16
7	HSLF	41.514	-2.9	12.206	45.721	0.060	0.1	ND	ND	17
8	HSL	33.480	-4.3	13.778	52.100	0.199	0.6	0.1	ND	33
9	HSL	32.609	-4.5	14.009	52.727	0.136	0.4	ND	ND	37
10	HSL cabin	9.438	-3.0	18.864	70.818	0.134	1.4	ND	ND	74
11	HSL vault	70.083	-4.5	6.049	23.582	0.277	0.4	0.1	ND	20
Lake sediments										
12	HSL (150)*	20.637	-6.4	10.816	67.699	7.346	35.6	ND	4.7(0.3)	552
Fumaroles										
13	MMF**	98.5	-4.6	~0.02	~1.10	—	~0	ND	5-7	13-17

* Locations of sites HSL, HSLF and MMF are shown in Fig. 1. Number in parentheses gives distance from respective tree-kill boundary in metres. Apart from MMF, [H₂] and [CH₄] are <10 p.p.m. and [H₂S] is <5 p.p.m.

† (±0.3%). For each sample, O₂, N₂ and CO₂ were analysed by high-precision gas chromatograph on the same injection onto the same column for a relative precision of ±0.1%, greatly minimizing the error in calculating O₂ depletion.

‡ ±0.1‰.

§ Defined as 0.26827N₂ (measured) - O₂ (measured), where 0.26827 is the O₂/N₂ ratio in air.

|| Defined as 100 × (oxygen depletion)/CO₂ (measured). This assumes a biogenic O₂-consumption: CO₂-production ratio of 1:1 in these soils.

¶ Abundance of ¹⁴C, expressed as a percentage of the 1994 level, taking 1994 level to be 113% modern carbon. Values in this column should represent the biogenic fraction of the CO₂ assuming fast turnover of carbon in these well drained forest soils. The available values support the assumed 1:1 conversion ratio. ND, not determined.

* Corrected for air using measured ⁴He/²²Ne/³⁶Ar ratios. Uncertainties in parentheses are 1σ. R/R_A is the ³He/⁴He ratio in the sample divided by the ³He/⁴He ratio in air.

* Sample collected just above low-water shoreline of Horseshoe Lake in October 1994.

** Listed values are from several recent analyses. Although the calculation of O₂ depletion at this site is not meaningful due, for example, to reaction between H₂S and O₂, the biogenic CO₂ at this barren area above tree-line is probably near zero.

areas, however, soils show normal temperatures and no visible signs of acid alteration.

Although oxygen deprivation⁷ may be involved in the tree kills, inhibition of root function by CO₂ (ref. 8) is probably more significant. The threshold concentration of CO₂ and time factor needed to cause mortality in these coniferous trees are not known, but the area presents a useful natural laboratory to study the effects of anomalous CO₂ increases on vegetation. Studies elsewhere have generally shown that elevated [CO₂] in the local atmosphere enhances photosynthetic rate (G. Koch, personal communication) and that some plants can adapt to permanently high soil [CO₂] (F. Miglietta, personal communication), but the sudden large increase in root-zone [CO₂], and forest die-off distinguish the Mammoth Mountain case. Dead-tree ages range up to ~250 yr, suggesting that these kill areas have not experienced a similar CO₂ anomaly for at least 250 yr.

Because Mammoth Mountain is a popular recreational area, the diffuse discharge of CO₂ has raised concerns over potential health hazards and effects on recreational facilities. In March 1990, a US Forest Service ranger reported symptoms of asphyxia when entering a small snow-covered cabin near Horseshoe Lake. Between 1990 and 1994 similar incidents were reported by other persons entering confined spaces, including subterranean utility vaults. Our preliminary survey of confined spaces in areas of known high CO₂ discharge near Horseshoe Lake found [CO₂] > 1% in campsite lavatories and small tents, 25% in a small cabin, and 89% in a utility vault of 0.6 m diameter and 1 m depth. All of these values exceed US health standards⁹ and the last two would be quickly lethal. The US Forest Service has recently closed the campsite at Horseshoe Lake until the potential hazard from CO₂ can be adequately assessed.

Snow accumulates to several metres depth over much of the mountain in winter, complicating CO₂ monitoring. Within tree-kill areas, concentrations of up to 60% CO₂ in snow have been measured in pits (1–2 m deep) shortly after excavation. Soil-gas samples collected through the snow from 11 permanently

installed access pipes at four locations show that the concentration of CO₂ increased by a factor of two to three between November 1994 and February 1995. These increases could be caused either by ice layers in the snow pack that inhibit gas transfer from the soil to the atmosphere¹⁰ or, possibly, by increased discharge rates from the source region.

The data in Table 1 provide clues to the gas source. The [CO₂] and $\delta^{13}\text{C}$ values of sample no. 1, collected 1,500 m outside a tree-kill boundary, fall within the ranges expected in a typical forest soil (~0.1–1.5% and -19 to -25‰, respectively) where CO₂ produced by respiration is steadily lost to the atmosphere by diffusion^{11,12}. The ¹⁴C activity confirms that the CO₂ in this sample is biogenic. The $\delta^{13}\text{C}$, ³He/⁴He and He/CO₂ values of the gas in tree-kill areas are near those of fumarole MMF (Fig. 1), and show magmatic characteristics³. The gas composition and $\delta^{13}\text{C}$ data also identify a component of magmatic CO₂ in healthy forest areas >100 m outside tree-kill boundaries (Fig. 3).

The CO₂ flow was measured in September 1994 at one site within the Horseshoe Lake tree-kill area where the [CO₂] in soil gas at 60 cm depth was 60%. A flow of 160 l m⁻² h⁻¹ was measured using an accumulation chamber placed on the bare soil surface. This site is typical of 50–75% of the total tree-kill area in terms of the degree of kill (nearly 100%) and [CO₂] (30–90%). In January 1995, a nearly identical flow was obtained in this same area at the base of a 1-m-deep snow pit (T. Rahn, personal communication). This flow is significantly higher than that reported for diffuse CO₂ emission from the flanks of the Mediterranean volcanoes Vulcano (≤2.64 l m⁻² h⁻¹, ref. 6) and Santorini (≤2.3 l m⁻² h⁻¹, ref. 13).

The four known tree-kill areas examined to date vary in area from a few to about 12 ha, and total over 30 ha (Fig. 1). A total flux of 1,200 tonnes per day (t d⁻¹) of CO₂ is obtained by assuming the measured CO₂ flow is characteristic of 20 of the >30 total ha. Considering that some outflow of magmatic CO₂ can reasonably be expected in areas of partial kill, in adjacent live-tree areas, and in the ~500 ha area above tree line, this

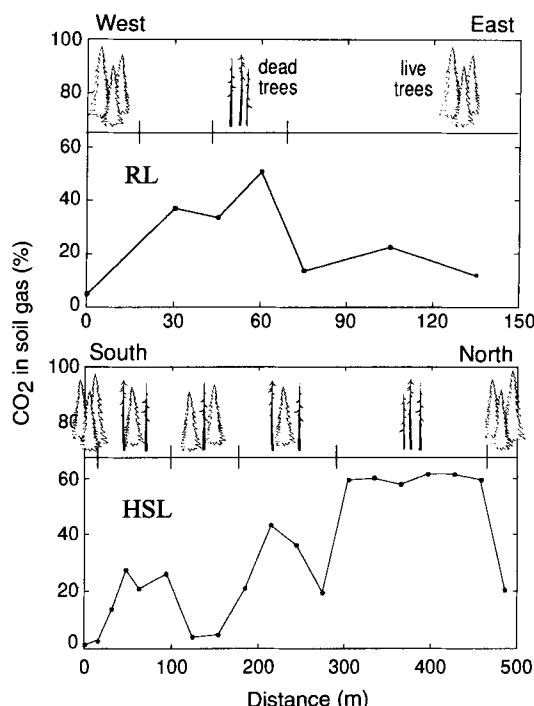


FIG. 2 Carbon dioxide concentrations in September 1994 at 0.6 m depth along transects shown in Fig. 1 (top panel, transect RL; bottom panel, HSL). Symbols at the top of each plot denote relative degree of tree mortality along the transects.

flux estimate is probably conservative for the entire Mammoth Mountain edifice.

At other volcanoes with known diffuse CO_2 fluxes, such as Mount Etna and Vulcano^{5,6}, voluminous high-temperature summit crater emissions provide a more noticeable indication of volcanic hazard. In contrast, Mammoth Mountain has only weak fumarolic activity (Fig. 1), which produces a very small fraction of the total gas flux. The massive vegetation kills are thus the most obvious indicators of possible magmatic unrest at depth. Interestingly, the estimated CO_2 flux of $1,200 \text{ t d}^{-1}$ is comparable to the summit crater fluxes reported for Kilauea, Augustine and Mt St Helens during recent periods of low-level eruptive activity^{14–17}.

To maintain a flux of $1,200 \text{ t d}^{-1}$ of magmatic CO_2 requires complete degassing of at least $0.025 \text{ km}^3 \text{ yr}^{-1}$ of magma, assuming that beneath Mammoth Mountain the magma is basaltic with a density of 2.7 g cm^{-3} and a maximum $[\text{CO}_2]$ of 0.65 wt% (refs 18, 19). This degassing rate is consistent with the rate of shallow dyke intrusion estimated from the seismic and deformation data for 1989–90 (refs 3, 20). The geophysical data indicate that shallow intrusive activity ceased after 1990. If CO_2 flux has been continuous over 1990–95 at rates comparable to $1,200 \text{ t d}^{-1}$, it is unlikely that all or most of this gas could have been supplied by a single intrusion of the size allowed by the geophysical data. Gas release from a single intrusive event in 1989–90 would also be expected to show a temporal decrease in the years following the intrusion, which is inconsistent with the available tree-kill evidence. However, long-period earthquake activity immediately southwest of Mammoth Mountain²¹ suggests continuing input of basaltic magma at mid-crustal depths since 1989. Degassing of deeper, multiple intrusions of basaltic magma could supply CO_2 at a constant or increasing rate. Alternatively, gas from such deep intrusions or from magma previ-

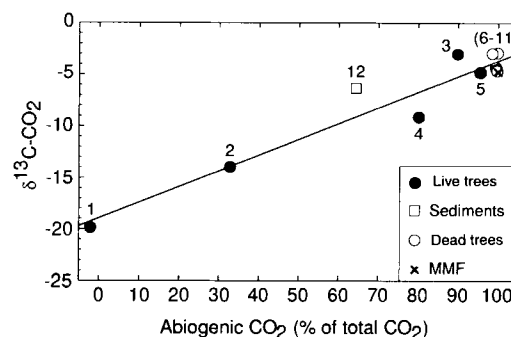


FIG. 3 Plot of $\delta^{13}\text{C}$ of CO_2 versus the fraction of CO_2 that is abiogenic, that is, the fraction not accounted for by 1:1 O_2 depletion (sample numbers and data from Table 1). The linear mixing trend (note that the small amount of atmospheric CO_2 can be ignored) demonstrates the presence of some magmatic gas in soil well outside the tree-kill areas, even in two cases (samples 2 and 4) where $[\text{CO}_2]$ is not elevated above background (sample no. 1). Sample no. 12, collected in exposed sediments where O_2 depletion is much greater than in the forest soils, also adheres reasonably well to the mixing trend.

ously emplaced within the edifice could gradually collect in a high-pressure volatile-rich zone that was breached during and following the 1989 intrusive event³. Cooling and chemical reaction in the storage zone might help to explain why the diffuse emissions are cold and contain none of the more reactive magmatic gases such as H_2 and H_2S . Geophysical evidence for a vapour zone under Mammoth Mountain has recently been described²².

Although the anomalous gas discharge carries uncertain implications for future volcanic activity, detection of the discharge has resulted in a heightened awareness of magmatic unrest at Mammoth Mountain, as distinct from the central part of Long Valley caldera where a 15-yr period of unrest was initiated by magnitude 6 earthquakes and uplift of the caldera floor in 1980 (refs 23, 24). Mammoth Mountain seems unique among volcanoes exhibiting signs of magmatic unrest in that there is at present no evidence of accompanying discharges of heat and acidic magmatic gases, or significant seismicity and deformation. The possibility that anomalous CO_2 discharge preceded or played a role in the 500 yr BP phreatic eruptions on the north side of Mammoth Mountain and the associated rhyolitic eruptions along the Inyo Craters volcanic chain is testable by ^{14}C measurements on individual rings in tree remains in the ejecta. Such information, along with additional gas-flux measurements and better constraints on transport processes, are needed to assess the volcanic hazard in this region. □

Received 10 May; accepted 28 July 1995.

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ACKNOWLEDGEMENTS. We thank R. A. Bailey and D. P. Hill for advice and counsel, T. Rahn for providing data, the Scripps Institution and members of the USGS Volcanic Hazards Program for their assistance. Carbon-14 work was performed under the auspices of US Department of Energy at Lawrence Livermore National Laboratory, Center for Accelerator Mass Spectrometry. Helium-isotope determinations by Lawrence Berkeley National Laboratory were partly supported by the Office of Basic Energy Sciences, Engineering and Geosciences Division of the US Department of Energy.

A plant-eating crocodyliform reptile from the Cretaceous of China

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WITH few exceptions, tooth shape among crocodyliform reptiles (Crocodylia of traditional use) is rather uniform¹. We report here on the presence of multicuspid molariform teeth in a remarkable new crocodyliform from the Lower Cretaceous of China, which may represent the first known herbivorous member of that group. The overall structure of these teeth is very similar to that of the postcanine teeth of tritylodontid synapsids and represents a particularly striking example of convergent evolution. It indicates back-to-front (proal) motion of the mandible produced by the posterior pterygoid muscle during jaw closing, much as in the extant tuatara, *Sphenodon*^{2,3}. Certain derived features indicate that the new Chinese crocodyliform is closely related to the Notosuchidae from the Cretaceous of Gondwana⁴. Its discovery thus casts further doubts on claims⁵ concerning an endemic Gondwanan tetrapod fauna during the Cretaceous.

Reptilia
Archosauria
Crocodyliformes

Chimaerasuchus paradoxus gen. et sp. nov.

Etymology. Generic name from Greek *chimaira*, mythical monster combining body parts of several animals, and *souchos*, Greek name for the Egyptian crocodile-headed god Sebek. Specific name Latin from Greek *paradoxos*, strange.

Holotype. Institute of Vertebrate Paleontology and Paleoanthropology, Academia Sinica, Beijing (IVPP) V8274, partial skeleton including snout and mandible, fifteen vertebrae, girdle and limb bones, and an osteoderm (Figs 1–3).

Type locality and horizon. Hill near the southern bank of Yangtze River, opposite Yichang city, Hubei province, China; Wulong Formation, Lower Cretaceous (Aptian–Albian)⁶. No other vertebrate fossils have been recovered from the site.

Diagnosis. Differing from all other known crocodyliforms in the possession of the following autapomorphies: two caniniform, procumbent premaxillary teeth; four molariform maxillary teeth, each with three longitudinal rows of recurved cusps; anterior end of jugal expanded laterally, overhanging posterior part of tooth row; angular with pronounced lateral projection posterior to suture between angular and dentary; splenial strap-like, apparently restricted to ventromedial surface of lower jaw. Additional possibly diagnostic features of currently equivocal status (because unknown in presumably related taxa) include: ilium without distinct blade and with rod-like preacetabular process; humerus with rounded fossa just distal to proximal head on lateral surface; osteoderm with peg-like ventrolateral process.

The formation of a complete secondary bony palate by the premaxillae and maxillae (Figs 1c, 2b), exclusion of the jugal from the antorbital fenestra (Fig. 1b), and elongation of the proximal carpals (Fig. 3f) support reference of *Chimaerasuchus* to the Crocodylomorpha^{7,8}. Diagnostic crocodyliform features include the broad ventromedial process of the coracoid (Fig. 3a) and the partial or complete exclusion of the pubis from the acetabulum by the anterior process of the ischium.

Chimaerasuchus has four closely appressed molariform teeth in each maxilla (Fig. 1c). Each molariform has three longitudinal rows of cusps that extend parallel to the midline (Fig. 1d). Each row comprises seven recurved cusps that decrease in size posteriorly so that the seventh is a mere cuspule. Each individual cusp bears a single concave, sharp cutting edge posteriorly. A cuspidate cingulum is developed along the anterolateral margin

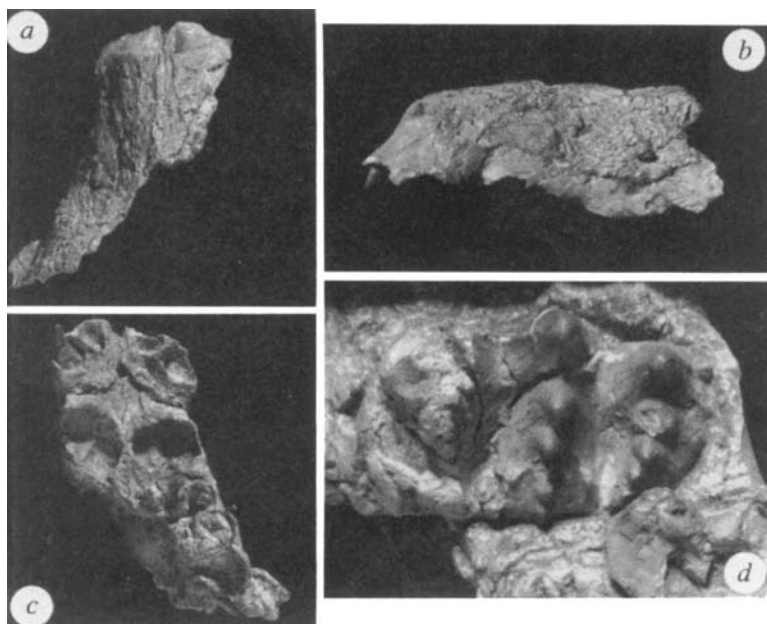


FIG. 1 *Chimaerasuchus paradoxus*, IVPP V8274 (holotype), from the Lower Cretaceous Wulong Formation of Hubei. Snout in dorsal (a), lateral (b) and ventral (c) views; left second maxillary tooth in occlusal view (d). Length of the snout fragment measured dorsally along the midline is ~4.3 cm; maximum width of tooth crown is ~1.6 cm. Most cranial bones bear pronounced external sculpturing comprising short ridges and grooves. The snout is relatively short and deep. The terminal external nares are confluent and face directly forward. A small antorbital fenestra is present. The complete maxillary tooth row on the left side comprises two teeth and two empty alveoli.

of the well preserved tooth crown. The molariform teeth are implanted in broad, shallow alveoli. The lower teeth are unknown, but the presence of more posterior molariform teeth is indicated by a broad and shallow alveolus for the posterior tooth on either side.

The articular facet of the jaw joint is distinctly longer than wide (Fig. 2c), indicating capacity for mandibular fore-and-aft motion⁹. The direction of the jaw movement during jaw closing is reflected by the curvature of the leading edge of the cusps of the molariform teeth¹⁰. Thus mandibular motion in *Chimaerasuchus* was back-to-front (proal), as in *Sphenodon*^{2,3} and inferred for a notosuchid crocodyliform from the Lower Cretaceous of Malawi⁹.

The maxillary teeth of *Chimaerasuchus* strikingly resemble the upper postcanines of the Tritylodontidae, highly derived non-mammalian synapsids of mainly Early and Middle Jurassic age¹¹. The cusps on the lower molariforms of *Chimaerasuchus*

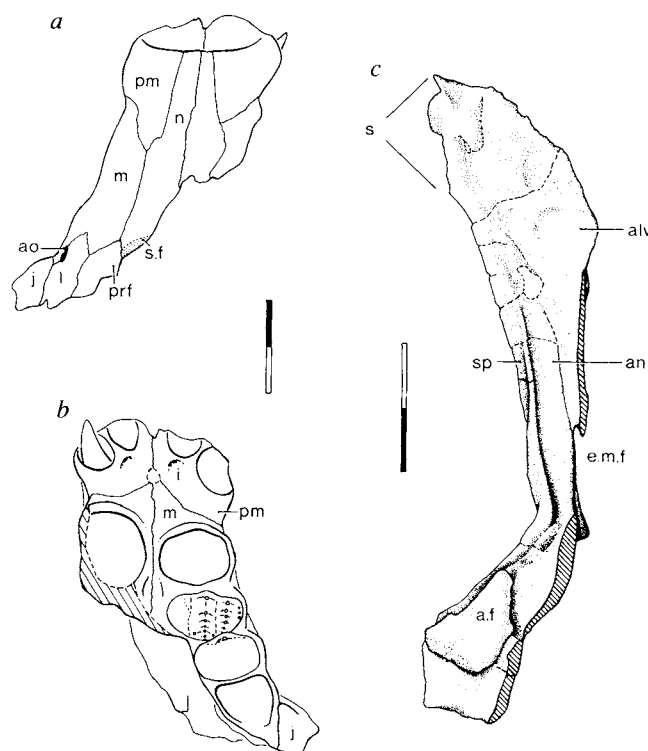


FIG. 2 *Chimaerasuchus paradoxus*, IVPP V8274 (holotype), from the Lower Cretaceous Wulong Formation of Hubei. a, b, Outline drawings of snout in (a) dorsal and (b) ventral views. c, Right mandibular ramus in dorsal view; note distortion due to extensive dorsoventral crushing. Scale bars, 2 cm. The articular facet of the jaw point is distinctly longer than wide and lacks a buttress-like posterior edge. The retroarticular process is broad and short. Abbreviations: a.f, mandibular articular facet of jaw joint; alv, posterior alveolus on dentary; an, angular; ao, antorbital fenestra; e.m.f, external mandibular fenestra; i, pit for incisiform anterior dentary tooth; j, jugal; l, lacrimal; m, maxilla; n, nasal; pm, premaxilla; prf, prefrontal; s, symphysis (incomplete); s.f, sutural facet for frontal; sp, splenial. Broken lines and cross-hatching denote fractures and broken surfaces, respectively.



FIG. 3 *Chimaerasuchus paradoxus*, IVPP V8274 (holotype), from the Lower Cretaceous Wulong Formation of Hubei. a, Left coracoid in lateral view; b, proximal portion of right humerus in lateral view; c, right ilium in lateral view; d, e, osteoderm in (d) ventral and (e) end views; f, left radiale in dorsal view; g, right ischium in lateral view. Scale bars, 2 cm (a–c, f, g) and 1 cm (d, e). The coracoid (a) and ischium (g) are typically crocodyliform. The lateral surface of the humerus (b) bears a rounded fossa just distal to the articular head. The combined length of the humerus and ulna exceeds the estimated length of skull. The ilium (c) lacks a dorsal blade but has a rod-like pre-acetabular process; its prominent supra-acetabular rim indicates a more erect posture than in extant crocodylians. The ischium (g) is relatively narrow distally and elongate. The osteoderm (d, e) has a peg-like ventrolateral process.

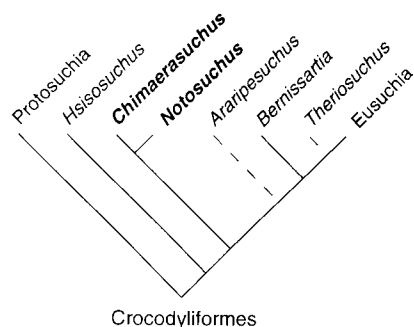


FIG. 4 Cladogram illustrating a hypothesis of the phylogenetic position of *Chimaerasuchus paradoxus*, derived from a numerical cladistic analysis (using PAUP) of 42 characters for 16 taxa of crocodyliforms (results in full to be presented elsewhere). Unequivocal synapomorphies for *Chimaerasuchus* and *Notosuchus*⁴ include: snout relatively broad and shorter than the remainder of the skull (determined in *Chimaerasuchus* from the length of the mandible); external nares vertical and facing directly forward; mandibular articular facet distinctly longer than wide; jaw joint in ventral position.

probably occluded in the grooves between the upper three rows. As in tritylodontids, the reversed concave cutting edges would have met during jaw closing and enclosed an ovoid space, which decreased in size during dynamic occlusion. This reversal of curvature in occluding teeth limited the area of tooth-to-tooth contact and thus served to maximize occlusal pressure between the teeth and food items at the contacts at any given time. However, mandibular motion was proal (back-to-front) rather than propalinal (front-to-back), and the presence of only a single posterior cutting edge, rather than paired crests, on each cusp indicates less extensive shearing in *Chimaerasuchus* than in tritylodontid synapsids. There are also no wear facets indicative of tooth-to-tooth occlusion. The structure of the maxillary molariforms is consistent with a specialized diet including fibrous material and may indicate at least facultative herbivory in *Chimaerasuchus*. The procumbent premaxillary (and probably anterior dentary) teeth were presumably involved in seizing food items.

The proal mandibular motion in *Sphenodon* is produced by the posterior pterygoid muscle^{2,3}. In extant crocodylians, the homologous muscle pulls the mandible forward and upward^{12,13}. However, as in *Sphenodon*, the jaw joint is ventral in position in *Chimaerasuchus* and the Malawi notosuchid. Consequently, the posterior pterygoid muscle in the latter two was probably similar in its orientation to that in *Sphenodon*. As in *Sphenodon*, mandibular motion in the two crocodyliform reptiles must have been proal during jaw closing because the anterior pterygoid muscle, along with the pterygoid flange, would have restricted mandibular movements to front-to-back motion during jaw opening.

Although the skeleton of *Chimaerasuchus* is not yet fully known, characters indicating close affinities to the Notosuchidae (Fig. 4) include the articular facet of the jaw joint that is much longer than wide⁹, the ventral placement of the jaw joint, and the terminal, directly forward-facing external nares.

Notosuchid crocodyliforms have been considered endemic to the Cretaceous of Gondwana^{4,5,9}. Along with other new fossils¹⁴, the discovery of *Chimaerasuchus paradoxus* casts doubts on claims⁵ that Gondwana had a distinctly endemic tetrapod fauna during the Cretaceous. □

Received 21 February; accepted 30 June 1995.

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ACKNOWLEDGEMENTS. Research supported by an NSERC Operating Grant to H.-D.S. Photographs by B. Boyle, drawings for Fig. 3 by J. Mulock.

Dimethyl sulphide as a foraging cue for Antarctic Procellariiform seabirds

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MANY Procellariiform seabirds make their living flying over vast expanses of seemingly featureless ocean waters in search of food. The secret of their success is a mystery, but an ability to hunt by smell has long been suspected^{1,7}. Here we present experimental evidence that Procellariiform seabirds can use a naturally occurring scented compound, dimethyl sulphide, as an orientation cue. Dimethyl sulphide has been studied intensely for its role in regulating global climate^{8,11} and is produced by phytoplankton in response to zooplankton grazing¹². Zooplankton, including Antarctic krill (*Euphausia superba*)¹³, are in turn eaten by seabirds and other animals¹⁴. Results from controlled behavioural experiments performed at sea show that many Procellariiforms can detect dimethyl sulphide, and that some species (for example, storm petrels) are highly attracted to it. To our knowledge, this constitutes the first evidence that dimethyl sulphide is part of the natural olfactory landscape overlying the southern oceans.

Experiments were conducted in sub-Antarctic waters near South Georgia during the Austral summer (January–March 1994). We chose this study area because Procellariiform species are numerous here, and the feeding habits of local species have been monitored for over two decades^{14,15}.

Our first set of experiments was designed to identify whether any Procellariiform species might be attracted to dimethyl sulphide (DMS). Our aim was to produce a down-wind DMS concentration that would be approximately the same as that which might naturally be encountered by a foraging petrel (nmol m⁻³ range)^{10,16,17}. We did this by deploying DMS-scented oil slicks on the ocean surface at ten different locations. Because surface slicks also presented visual cues to seabirds, DMS slicks were always paired with unscented ‘control’ slicks. We reasoned that if birds used DMS as a foraging cue, more birds should be attracted to DMS than to control slicks. At some locations, we also compared the response of birds to DMS with their response to cod liver oil, a well known olfactory attractant^{1,3,4,6,7}.

Results from our paired-slick experiments indicate that DMS is a potent olfactory attractant to many Procellariiform species, including white-chinned petrels, prions and two species of storm petrels (Fig. 1)¹⁸. Wilson’s storm petrels, for example, were sighted more than twice as often flying into DMS slicks than into control slicks. Indeed, when we plot the temporal response profiles of Wilson’s storm petrels to both DMS and cod-liver oil slicks (Fig. 2), we find that the outcomes are remarkably similar suggesting that DMS is as potent an olfactory attractant

as cod liver oil to this species. Just as noteworthy, however, is the absence of any response to DMS on the part of albatrosses or cape petrels: these species arrived at both DMS slicks and control slicks with the same frequency (Fig. 1).

In a second set of experiments, we presented birds with aerosol plumes of either DMS or water and monitored their flight behaviour. This approach allowed us to test birds' responses to DMS without presenting them with visual cues. Our hypothesis was that if DMS constituted a foraging cue, then birds likely to be attracted to this odour would also increase their turning rate in an attempt to locate the source^{16,18,19}. For these experiments, either odour (DMS) or control (water) plumes were aerosoled off the ship's stern while a pair of observers monitored the flight behaviour of the birds. White-chinned petrels and black-browed albatrosses were chosen for this investigation because these species showed distinct responses to DMS-scented slicks: white-chinned petrels were attracted to DMS, whereas black-browed albatrosses were not. We therefore predicted that white-chinned petrels would increase their turning rate in response to DMS, whereas black-browed albatrosses would not.

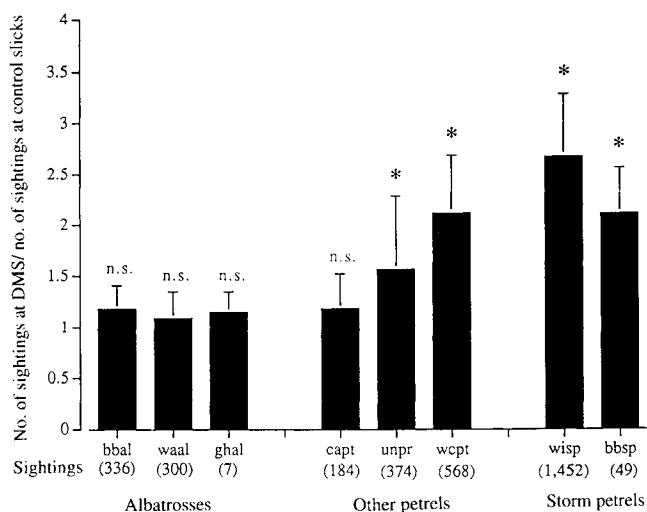


FIG. 1 Relative attraction of Procellariiform seabirds to DMS-scented oil slicks. The ratio of bird sightings at DMS slicks to sightings at control slicks was calculated for each pair of slicks. The data plotted represent means and standard errors of these 10 ratios. A value of one is expected if birds show identical responses to control and DMS-scented slicks. Significant differences (G-test, $P < 0.05$) are marked with asterisks. The total numbers of sightings during 10-min observation periods for the 10 paired slicks are listed below the species codes. Abbreviations: bbal, black-browed albatross (*Diomedea melanophrys*); waal, wandering albatross (*Diomedea exulans*); ghal, grey-headed albatross (*Diomedea chrysostoma*); capt, cape petrel (*Daption capense*); unpr, unidentified prion species; wcpt, white-chinned petrel (*Procellaria aequinoctialis*); wisp, Wilson's storm petrel (*Oceanites oceanicus*); bbsp, black-bellied storm petrel (*Fregetta tropica*).

METHODS. While the ship was positioned into the wind, a control, DMS- or cod-liver-oil-scented slick (Fig. 2 legend) was deployed, and typically drifted about 100 m from the vessel, allowing easy observation of approaching birds. Birds were counted if they (1) flew upwind over the slick within approximately 1 m of the surface, (2) alighted, or (3) pattered on the slick. Observations were made at 1-min intervals starting 2 min before deployment. Experiments were conducted as blinds: no-one involved in data collection knew the identities of the slicks, and the order of presentation was assigned randomly for each location. Data were logged on a hand-held computer so that the exact time of each event was automatically recorded. Slicks were tested one at a time and generally dissipated within 20 min; presentations were separated by at least 1 h. Experiments were conducted northwest of South Georgia (between 54°15' S, 38°00' W and 51°15' S, 41°15' W). Half (that is, 5 paired slicks for DMS and 3 paired slicks for cod-liver oil) were performed over deep water (>1,000 m), whereas the other half were performed over water ~100 m deep.

Our turning frequency results were consistent with the hypothesis we generated from our paired slick experiments (Fig. 3). White-chinned petrels turned 36% more frequently in the presence of DMS aerosol plumes than they did in response to control water plumes ($N_1 = 35$ birds, water plume; $N_2 = 34$, DMS plume; $U = 440$, $P < 0.03$, Mann-Whitney U -test)¹⁹. In contrast, turning behaviour in black-browed albatrosses was not affected by DMS ($N_1 = 20$ birds, water plume; $N_2 = 30$ birds, DMS plume; $U = 285$, $P > 0.40$), suggesting that these birds either did not detect DMS aerosols, or that they were not motivated to alter their behaviour in a fashion that would focus their activity in regions of high DMS concentrations^{16,20}.

The finding that different species of Procellariiforms showed variable responses to DMS is consistent with many aspects of their feeding behaviour. Albatrosses, which did not respond to DMS slicks any more than to control slicks, are large, noticeable birds, and frequently hunt visually^{14,21}. Like cape petrels, they often forage by spotting and exploiting mixed-species feeding aggregations of seals, whales and other visibly conspicuous seabirds²². Storm petrels, white-chinned petrels and prions, however, were consistently attracted to DMS and their ecology¹⁴ and retinal anatomy²³ suggests that they may be less likely to exploit visual cues as readily while foraging. These species have been shown to occur at much lower frequencies than either albatrosses (black-browed and grey-heads) or cape petrels in the mixed-

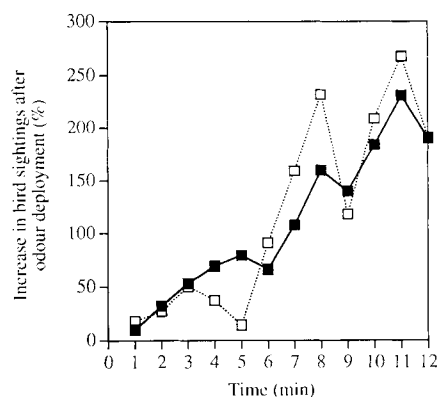
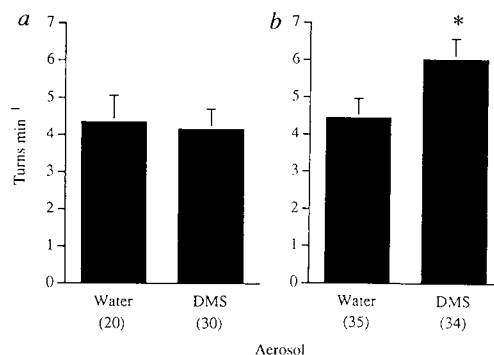


FIG. 2 Response of Wilson's storm petrels to DMS (filled squares) and cod liver oil (open squares) scented slicks. Control responses have been subtracted. Data are combined from 10 paired trials for DMS (that is, 10 DMS-control slick pairs) and six paired trials for cod liver oil. Data are normalized to the numbers of birds that were present in a visual area 200 m around the ship before each slick was deployed. Odour presentations: DMS (200 mM DMS (0.5 mol total, Aldrich Chemical) in 2.5 litres vegetable oil), cod liver oil (380 ml cod liver oil (Squib Pharmaceuticals) diluted to 2.5 l in vegetable oil) and control slicks (2.5 l vegetable oil) were prepared from stock solution ~1 h before experiments. As the ability to predict turbulent plume dynamics is poor, we adapted a simple sector model derived from empirical sampling of turbulent odour plumes to approximate average odour profiles downwind of slicks^{16,19}. This conservative approach suggests that (1) the odour disperses as a cone-shaped plume downwind of the slick with edges 20 deg from the axis; (2) the odour is dispersed continuously and rapidly (~300–600 m min⁻¹) downwind of the slick, reflecting high wind velocities during experiments (~10–20 knots; 4.6–9.4 m sec⁻¹); and that (3) this establishes an odour gradient extending kilometres downwind of the slick over the time course of the experiment (10 min). We used this model to calculate the theoretical maximum average concentration that a bird might encounter traversing a plume extending 1 km downwind of the slick. To do this we calculated what would happen if all the odour deployed was concentrated in a plume extending 1 km downwind of the slick. Although we recognize that turbulent odour plumes are not homogeneous, even this exaggerated scenario yields an average concentration of <10 nmol m⁻³, well within natural levels measured in the Antarctic (7.0–9.8 nmol m⁻³)¹⁷. Based on published estimates of flight velocity³⁰, we calculate that the maximum distance storm petrels could have travelled to reach scented slicks in the time allowed was 4 km.

FIG. 3 The influence of DMS aerosols on turning behaviour. Black bars indicate turning rates for *a*, black-browed albatrosses and *b*, white-chinned petrels, in response to water or DMS aerosols. Significant differences (Mann–Whitney *U*-test; see text) are marked with asterisks. Numbers of individual birds observed for each group are indicated in parentheses. For each observation, an individual bird was identified in a predefined visual zone set ~ 300 m at a 45-deg angle off the stern. Once a bird was spotted, the observer dictated the direction of the flight path to a partner, who entered the data into a portable computer. A new direction was entered each time the bird turned by 90 deg or more. Observers followed birds for up to 45 s, or until they entered the wake of the ship. Although treatments were always paired, the observers had no knowledge of what stimulus (DMS or water) was being presented at any given time, and were not in visual (or olfactory) contact with the person deploying the odorant. These experiments were conducted in high wind relative to slick experiments (~ 20 – 30 knots; 9.4 m sec^{-1} – 13.9 m sec^{-1}). As the ship headed upwind (6.8 – 10.2 km hr^{-1}), a 10-s aerosol spritz of either DMS (0.02 mol per spritz) or water was delivered once every 2 min for 40 min. Aerosols were delivered from a height of 6 m above the ocean's surface using a hand-held aerosol sprayer (Preval, Inc.). At 50 min the stimulus presentation was alternated. Again, odour plumes were designed as outlined above (Fig. 2 legend), except that we presented birds with an intermittent odour gradient aerosoled downwind. As animals tend to turn in response to abrupt changes in odour concentration, we reasoned that an intermittent presentation would maximize the likelihood of seeing such behaviour. As discussed in Fig. 2 (legend), we assumed that each 0.02 M DMS spritz would disperse as a cone-shaped plume downwind of the slick, with edges 20 deg from the axis. Estimates based on relative wind velocities suggested that aerosols would be transported 1 km or more within 2 min. As a conservatively high estimate, however, we calculated an average DMS concentration based on a 300 m transport distance to be about 10 nmol m^{-3} .



species feeding aggregations that have been intensively studied near South Georgia²². In addition, all are cryptically coloured, visually inconspicuous birds, and often forage at night when olfactory cues might be especially valuable^{21,24}. Nevertheless, all the Procellariiform species tested here possess a strikingly large and complex olfactory anatomy²⁵, suggesting that, whether or not birds can detect DMS, this particular odourant may be a more compelling foraging cue for some species than for others.

How might an ability to detect DMS be advantageous to a seabird? Marine DMS is a byproduct of the metabolic decomposition of dimethyl sulphonioacetate (DMSP) in marine phytoplankton (most notably *Phaeocystis pouchetii*), and this process is dramatically accelerated during grazing by zooplankton^{12,26}. The ability to detect and recognize DMS as a potential feeding cue could thus be advantageous in locating and exploiting zooplankton-rich areas, especially as natural DMS emissions are not strictly ephemeral, but can persist for hours

or even several days¹⁷. In addition, because DMS concentrations tend to be highest in surface seawater associated with oceanic features such as upwelling zones and shelf waters¹¹, these features, reflected in the atmosphere as elevated DMS profiles, could present a navigable olfactory landscape to seabirds²⁷. This is an intriguing idea as Procellariiforms live highly pelagic life styles, and are also known for seasonally migrating extreme distances by navigational mechanisms that are not well understood²¹. The use of odour gradient maps has been proposed for pigeon homing, but the identification of natural biogenic aromatics has proven elusive^{27–29}. Our study now adds an additional clue to understanding how natural odour landscapes aid vertebrate search behaviour, first by identifying DMS as one of the naturally occurring components of the olfactory landscape overlying the Southern oceans, and second, by experimentally demonstrating that members of at least one vertebrate group, the Procellariiform seabirds, are capable of exploiting this natural biogenic aromatic to guide their foraging behaviour. □

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ACKNOWLEDGEMENTS. We thank T. Bates for encouragement and for introducing us to DMS; B. Anson, L. Clark, D. Grunbaum, D. Lenzi, P. Prince, W. Roberts, E. Silverman and M. Whitehouse for discussion, technical assistance or proof reading; the scientists, officers and crew of the RRS *James Clark Ross* (1994 Predator/Prey Cruise: JR06) and J. Croxall, P. Prince, M. White and the British Antarctic Survey for their help with this project. Support for this research was provided by an NSF Polar Programs grant to P.K., R.V. and J. Wingfield.

Molecular mechanism for an inherited cardiac arrhythmia

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In the congenital long-QT syndrome, prolongation of the cardiac action potential occurs by an unknown mechanism^{1,2} and predisposes individuals to syncope and sudden death as a result of ventricular arrhythmias³. Genetic heterogeneity has been demonstrated for autosomal dominant long-QT syndrome by the identification of multiple distinct loci^{4,5}, and associated mutations in two candidate genes have recently been reported^{6,7}. One form of hereditary long QT (LQT3) has been linked to a mutation⁷ in the gene encoding the human heart voltage-gated sodium-channel α -subunit (*SCN5A* on chromosome 3p21)⁸. Here we characterize this mutation using heterologous expression of recombinant human heart sodium channels. Mutant channels show a sustained inward current during membrane depolarization. Single-channel recordings indicate that mutant channels fluctuate between normal and non-inactivating gating modes. Persistent inward sodium current explains prolongation of cardiac action potentials, and provides a molecular mechanism for this form of congenital long-QT syndrome.

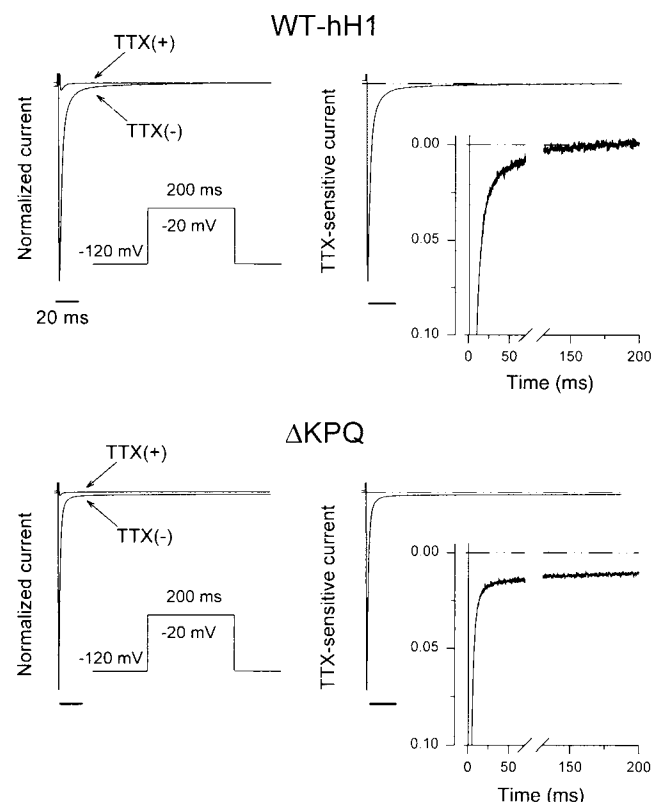
Figure 1 shows two-electrode voltage-clamp recording from *Xenopus laevis* oocytes expressing either wild-type (WT) human heart Na⁺ channel (hH1) or the LQT3 mutation designated Δ KPQ (in-frame deletion of residues Lys 1,505, Pro 1,506, Gln 1,507 in *SCN5A*). Normalized tracings of both Δ KPQ and wild type demonstrate similar rapid macroscopic inactivation and each exhibit a similar biexponential time course. Remarkably, we found that time constants for current decay were decreased in the Δ KPQ mutant (WT-hH1: $\tau_{\text{fast}} = 1.47 \pm 0.11$ ms,

$\tau_{\text{slow}} = 8.59 \pm 0.71$ ms, $n = 18$; Δ KPQ: $\tau_{\text{fast}} = 0.98 \pm 0.07$ ms, $\tau_{\text{slow}} = 5.40 \pm 0.55$ ms, $n = 13$; $P < 0.01$; data are means $\pm$ s.e.m.) not increased as was predicted⁷ on the basis of deletion mutagenesis work performed with rat brain IIA Na⁺ channels⁹. However, unlike WT-hH1, currents recorded in Δ KPQ-expressing cells did not decay completely within a 200-ms depolarization. This persistent current represented less than 5% of the peak inward current at -20 mV, and was blocked by 30 μ M tetrodotoxin (TTX) (Fig. 1, insets). Another surprising feature of the Δ KPQ mutant was a significant shift in the membrane potential at which 50% of the current is inactivated (WT: -74.6 ± 0.9 mV, $n = 16$; Δ KPQ: -80.4 ± 1.1 mV, $n = 19$; $P < 0.002$). The slope factor for this relationship was not changed (WT: 4.35 ± 0.13 mV, $n = 16$; Δ KPQ: 4.22 ± 0.16 , $n = 19$). In LQT cardiac myocytes, this shift in steady-state inactivation midpoint would decrease the availability of the mutant channel relative to WT-hH1 at the resting membrane potential. There was no observable difference in the kinetics of recovery from inactivation between WT and mutant Na⁺ channels (data not shown). The same qualitative results were observed in one other independent mutant clone.

To understand fully the functional disturbance conferred by the Δ KPQ mutation, we performed single-channel analysis on both wild-type and mutant Na⁺ channels. Figure 2 shows data from experiments using inside-out excised membrane patches. The WT-hH1 channels opened briefly (< 1 ms) only once or a very few times during depolarization and these openings always occurred within the first 20 ms (Fig. 2a, c). Single-channel records obtained from oocytes expressing Δ KPQ channels demonstrated a similar frequency of initial openings, but mutant channels would occasionally exhibit multiple reopenings ('bursts') occurring much later than 20 ms (Fig. 2b, d). This intermittent reopening behaviour gave rise to a small sustained inward Na⁺ current, as revealed by the ensemble average (Fig. 2e, f). In data obtained from three separate membrane patches (total 400 sweeps), bursting occurred in $3.5 \pm 1.3\%$ of sweeps recorded from Δ KPQ-expressing cells, but was not seen in WT-

FIG. 1 Sodium currents recorded in *Xenopus* oocytes expressing either WT-hH1 (top) or Δ KPQ (bottom). Representative current traces obtained in the presence or absence of 30 μ M TTX and recorded during a 200-ms test depolarization to -20 mV from a holding potential of -120 mV (left panels). Peak current amplitudes are normalized to that obtained in the absence of TTX. The result of subtraction of currents obtained in the presence from those in the absence of TTX are shown in the right panels. The same subtracted data are shown on an expanded scale in the insets. Sodium-current expression was similar in WT and Δ KPQ RNA-injected oocytes.

METHODS. Deletion of amino-acid residues 1,505–1,507 in the recombinant human heart Na⁺-channel α -subunit (hH1) was accomplished by overlap extension polymerase chain reaction mutagenesis¹⁹, and the final product was assembled into the plasmid pSP64T-hH1 (ref. 20). Multiple independent recombinants were selected, analysed by digestion with restriction enzymes, and the mutant inserts sequenced in their entirety. Clones were identified that were free of polymerase errors and used for electrophysiological studies. *In vitro* transcription reactions, microinjection of *Xenopus* oocytes, and two-electrode voltage-clamp recording were done as described²¹. Oocytes were injected with 20 ng RNA.



hH1 under the same experimental conditions. The single most striking feature of the Δ KPQ mutant channels was their propensity to reopen repeatedly at times much later than 20 ms, in contrast to WT-hH1. We specifically compared the late-opening behaviour of the mutant and wild-type channels occurring between 30–200 ms after the onset of the test depolarization. During this time interval, the probability of WT-hH1 channel

openings was zero (data not shown). In contrast, mutant channels displayed a substantial open probability throughout the period (Fig. 3a). The abnormal gating behaviour displayed by the Δ KPQ channel was not associated with a change in the duration of single-channel openings (Fig. 3b, c).

We used computer simulations of Na^+ -channel gating based upon multistate Markov gating models to test specific hypotheses put forth to explain the abnormal behaviour of the Δ KPQ channel (Fig. 4). Our data can be used to exclude the mechanisms shown in Fig. 4b–d. Therefore, we considered an alternative hypothesis: modal gating (Fig. 4e, f). Modal gating appears to be a general kinetic feature of mammalian Na^+ channels^{10,13}, but in contrast to the behaviour of normal cardiac Na^+ channels, which exhibit rare (<0.1%) transitions into a non-inactivating gating mode¹⁴, the Δ KPQ mutant entered this mode in 3.5% of depolarizations. A simplified model for this hypothesis is shown in Fig. 4e and f. Here the channels gate normally most of the

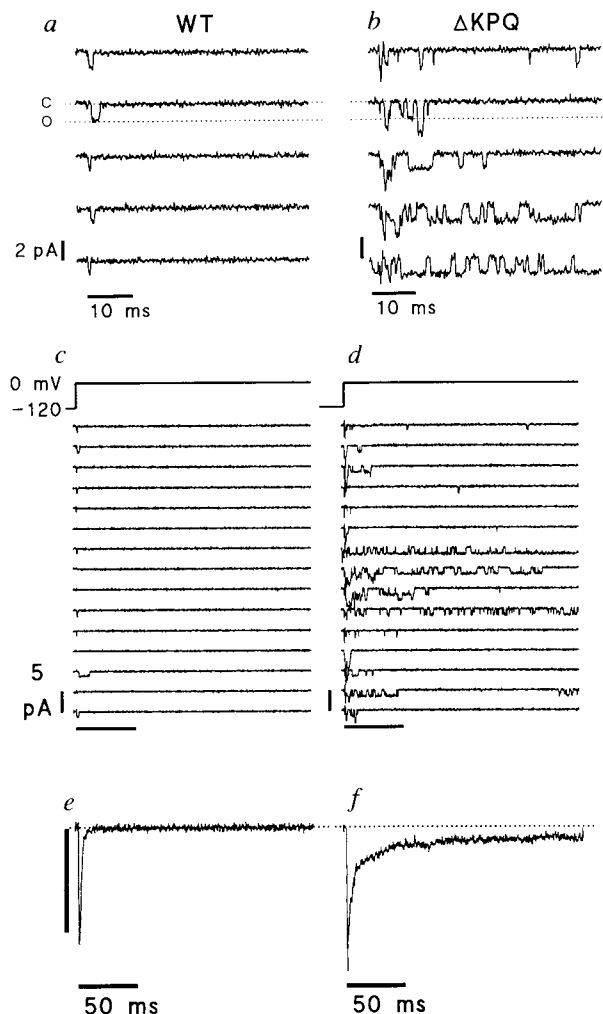


FIG. 2 Single-channel recordings from Na^+ channels in inside-out membrane patches excised from *Xenopus* oocytes during 200-ms duration voltage-clamp steps. **a**, Selected recordings of unitary WT-hH1 Na^+ channel current (data from the first 50 ms are shown). **b**, Selected recordings of unitary Δ KPQ mutant Na^+ channel current (data from the first 50 ms are shown). **c**, Records from WT-hH1 channels during 200-ms voltage-clamp steps. **d**, Records from Δ KPQ channels during 200-ms voltage-clamp steps. Time calibration bars in **c** and **d** represent 50 ms. **e**, Ensemble average of WT-hH1 Na^+ channel current. The vertical axis was scaled to open probability (P_{open}) by dividing the ensemble average current by the unitary current (i) and the number of channels in the patch (N). Vertical calibration bars represent P_{open} of 0.2. **f**, Ensemble average of Δ KPQ mutant Na^+ -channel current.

METHODS. Oocyte vitelline membranes were removed by hypertonic stripping²². Single Na^+ channels were recorded in the inside-out configuration²³ and analysed as described²⁴. Currents were recorded during voltage steps to 0 mV from a holding potential of -120 mV. Single Na^+ -channel currents were filtered at 2 kHz and digitized at 10 kHz. Patch pipettes were pulled from borosilicate glass and filled with (in mM) 145 NaCl, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, pH 7.35. The bath solution ('intracellular') contained (in mM) 110 CsF, 10 NaF, 20 CsCl, 2 EGTA, 10 HEPES, pH 7.35.

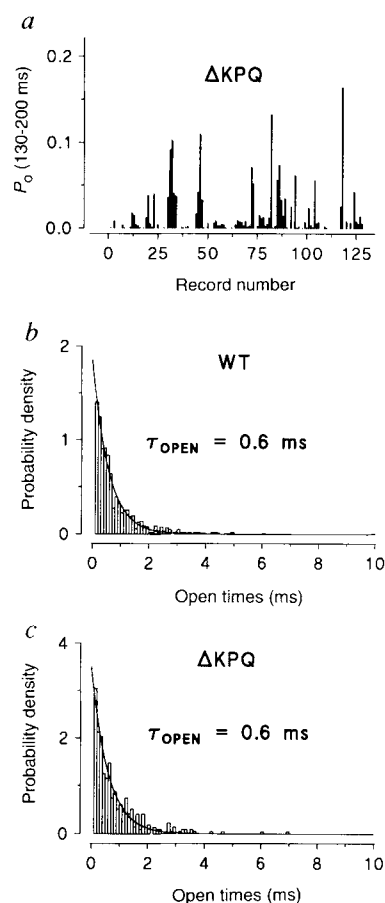


FIG. 3 Open-probability diaries and open-time distributions. **a**, Open probability in each record for Δ KPQ mutant channels 30–200 ms after a voltage step to -20 mV. No openings of WT-hH1 were observed during the interval 30–200 ms ($P_{\text{open}} = 0$). **b**, Distribution of open times for WT-hH1 recorded at -20 mV. **c**, Distribution of open times for Δ KPQ mutant recorded at -20 mV.

METHODS. Unitary current was estimated from all points amplitude histograms and open times were measured using a 50% threshold-crossing criterion as described²⁴. Open-time distributions were made by sorting open durations into bins and plotting (or fitting) the number of openings per bin as a function of open time. Probability density was calculated by dividing the number of events in each bin by the total number of events and the bin width. Diaries were made by integrating the current ($N \times P_o \times i \times s$) in each record and dividing by the single channel current (i), the number of channels in the patch (N) and the duration of the record.

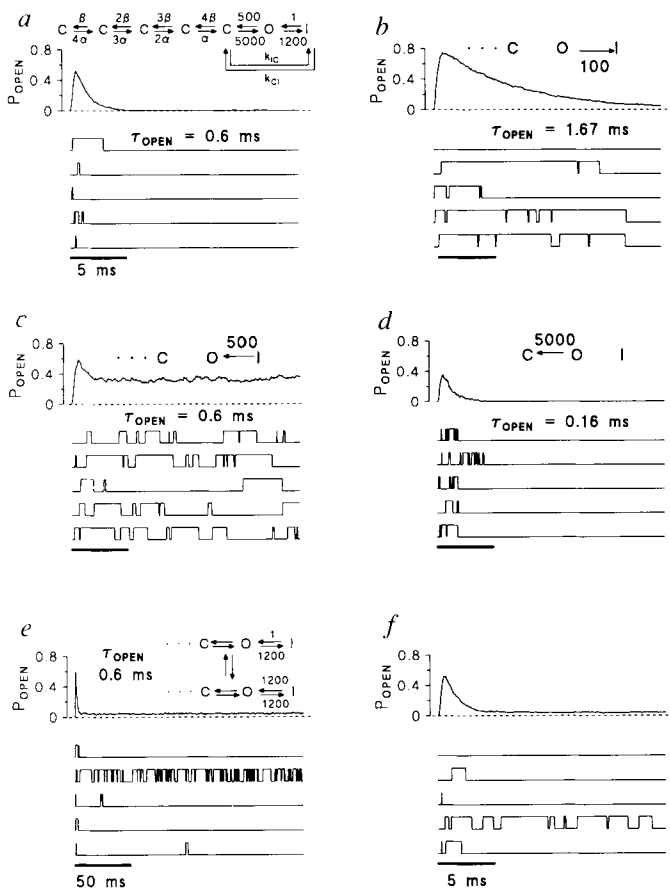


FIG. 4 Predicted behaviour of Na^+ -channel gating based on multistate Markov gating models. For simplicity, we adopted a Na^+ -channel model having a single open state²⁵. **a**, WT gating model. In alternative models (**b–f**), only changes from the WT-model rate constants are shown. Predicted open times are shown in each panel. **b**, A 12-fold decrease of the rate constant entering the inactivated state causes slower macroscopic current decay and prolonged open times. **c**, A 500-fold increase of the rate constant leaving the inactivated state causes channels to return readily from the inactivated state. There is no effect on the open times, but the channels open and close continuously. Smaller increases in this rate constant did not cause a sufficient number of reopenings. **d**, An increase of the rate constant returning to the first closed state from the open state causes numerous open–closed transitions. However, this mechanism also shortens the open times. **e**, **f**, Modal gating model. The channel randomly enters an alternate gating mode with a probability of 0.05. In the alternate gating mode, the inactivation rate constant was unchanged. The rate constant for returning to the open state from the inactivated state was increased from 1 to 1,200 s^{-1} . All rate constant are given in units of s^{-1} .

METHODS. WT-hh1 was modelled as a series of closed states preceding a single open state followed by an inactivated state; τ_{open} is the mean open time. Typical single-channel openings and the ensemble open-channel probability are shown as functions of time. Forward rate constant α was 10,000 s^{-1} ; reverse rate constant β was 100 s^{-1} ; k_{ic} and k_{ci} are the rate constants going to or returning from the inactivated state to the first closed state, respectively; k_{ic} was set to 1,000 s^{-1} and k_{ci} was adjusted to enforce microscopic reversibility.

time, but occasionally enter an altered gating mode where the channel bursts for prolonged periods. Substantial evidence implicates the cytoplasmic region linking repeat domains 3 and 4 (ID3-4) in Na^+ -channel inactivation^{15,16}. It has been postulated that this region forms the inactivation gate of the Na^+ channel by forming a lid that closes over the open ion-conducting pore, causing inactivation. One possible molecular mechanism for the altered gating caused by the ΔKPQ deletion is that the flexibility of the ID3-4 is modified, which effectively increases the unbinding rate of the ID3-4 from its docking site.

Long-QT syndrome is the first recognized inherited myocardial ion-channel disease. Our demonstration of a disturbance in Na^+ -channel function is necessary and sufficient proof that ΔKPQ can cause the LQT phenotype. In cardiac myocytes, the

observed abnormal Na^+ -channel gating will result in a small fraction of persistent inward Na^+ current flowing during the plateau phase of the cardiac action potential. The plateau is normally maintained by a delicate balance between inward and outward currents¹⁷, and repolarization occurs as activating outward currents prevail over inactivating inward currents. Dysfunction of an outward potassium current is presumed to cause one form of congenital LQT (LQT2)¹⁸. In LQT3, the sustained inward current generated by ΔKPQ will prolong action potential duration by shifting this balance in favour of inward current, and cause lengthening of the Q–T interval. This sustained inward Na^+ current might also promote other inward currents (calcium current, electrogenic sodium–calcium exchange), thus amplifying the QT-prolonging effect of the mutation. □

Received 2 May; accepted 28 June 1995.

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ACKNOWLEDGEMENTS. We thank D. Roden and D. Snyders for comments on the manuscript, and J. V. Kodali for technical assistance. This work was supported by the NIH and the American Heart Association. P.B.B. is an Established Investigator of the American Heart Association. A.L.G. is a Lucille P. Markey Scholar.

Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin

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THE neuronal growth cone is thought to be the site of decision making in nerve growth and guidance^{1,2}. One likely mechanism of how the growth cone translates various extracellular cues into directed motility involves rises in intracellular calcium. A variety of physiological cues, such as adhesion molecules and neurotransmitters, increases intracellular calcium¹, and artificial manipulations of growth cone calcium levels affect growth cone morphology and neurite outgrowth³. The molecular events downstream of calcium fluxes are incompletely understood. Here we show that calcineurin, a protein phosphatase enriched in growth cones that is dependent on calcium ions and calmodulin⁴, functions in neurite outgrowth and directed filopodial motility in cultured chick dorsal root ganglia neurons. Cyclosporin A and FK506, inhibitors of calcineurin⁵, delayed neuritogenesis and inhibited neurite extension. Chromophore-assisted laser inactivation of calcineurin in regions of growth cones causes localized filopodial and lamellipodial retraction and influences the direction of subsequent outgrowth. We suggest that a spatial distribution of calcineurin activity within the growth cone can regulate motility and direct outgrowth.

We characterized the effects of the immunosuppressants cyclosporin A (CsA) and FK506 on neurite outgrowth in chick dorsal root ganglia (DRG) neurons cultured on laminin. At a concentration of 1 μ M CsA or FK506, 4-h drug treatments decreased the production of neurites by about threefold (Fig. 1a). At 24 h, drug-treated cells had an equivalent number of neurites as control cells, but their neurites were much shorter (data not shown). To distinguish possible effects on neurite extension from those on neuritogenesis, drug treatments were delayed until 4 h after the cells were plated. By this time, about 60 per cent of the neurons have produced neurites (Fig. 1a). Delayed treatment of CsA or FK506 for 4 h (8 h total culture time) shifted the length distribution of neurites and significantly decreased the median neurite lengths (Fig. 1b). FK506 may be more potent than CsA in inhibiting neurite extension because of the higher concentrations of its receptor protein FKBP in neural tissues⁶ or the lower inhibition constant (K_i) of the FK506·FKBP complex for calcineurin⁷. Treatment with 1 μ M rapamycin, a ligand of FKBP but not an inhibitor of calcineurin, did not perturb neuritogenesis, and in fivefold excess, rapamycin antagonized the effects of FK506 (Fig. 1a). Although rapamycin alone inhibited neurite extension, excess rapamycin antagonized the more potent inhibition of FK506 on neurite extension (Fig. 2b). The similar effects of CsA and FK506 suggest that calcineurin, the common target of the two drugs⁵, regulates neuritogenesis and neurite extension. The rapamycin-sensitive FRAP/RAFT⁸ pathway may be involved in neurite extension but not neuritogenesis. The antagonism between FK506 and rapamycin distinguishes calcineurin's role in neurite outgrowth from FKBP's role in sensitizing neurons to nerve growth factor⁸. Our data extend on the observation that a peptide inhibitor of calcineurin inhibited polarized axon elongation in cerebellar macroneurons⁴. Calcineurin's role in neurite outgrowth (manifested over a course of hours) may involve regulating cytoskeletal dynamics⁴ and changing gene

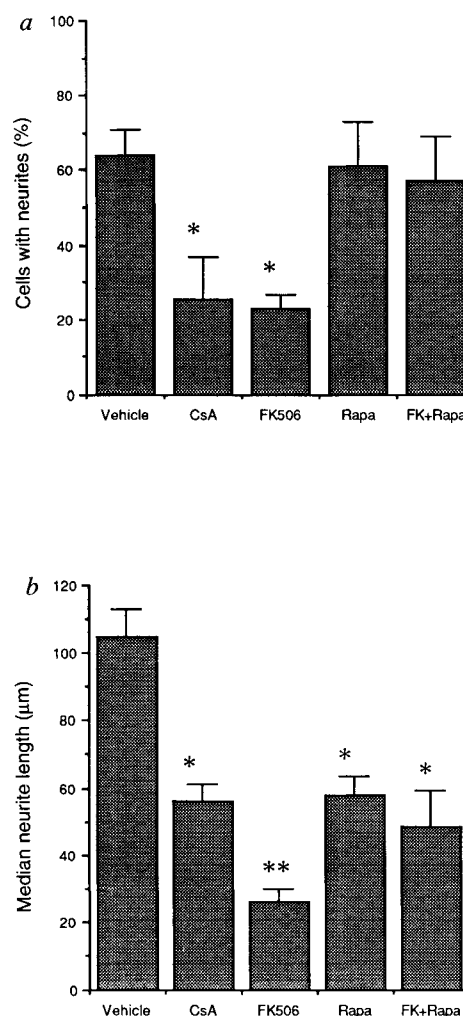


FIG. 1 CsA and FK506 delay neuritogenesis and inhibit neurite extension. **a**, Neuritogenesis is significantly inhibited by 1 μ M CsA or FK506. CsA, FK506 or rapamycin (1 μ M alone or 5 μ M with 1 μ M FK506) were added at the time of plating. Data shown are means $\pm$ s.d. After 24 h, drug treatment produced no significant difference in the percentage of cells with neurites, which ranged between 85% and 90% for all categories (95% confidence interval for vehicle alone: 74%, 96%). Drug degradation is unlikely because others have observed drug effects lasting for 24 h or longer²⁴. * Significantly lower than vehicle, $P < 0.002$. **b**, Effect of 1 μ M CsA or FK506 on neurite extension. DRG neurons were treated with 1 μ M CsA, FK506 or rapamycin (1 μ M alone or 5 μ M with 1 μ M FK506) at 4 h after plating for 4 h more. Data shown are average median neurite lengths $\pm$ s.d. For comparison, the median neurite length of a 4 h control culture is 70 ± 6 μ m. CsA or FK506 caused no observable change in cell number or number of neurites per cell. * Significantly lower than vehicle, $P < 0.002$. ** Significantly lower than all other values, $P < 0.002$.

METHODS. DRG neurons from E11 chick embryos were isolated and cultured on poly-L-lysine and laminin-coated coverslips with NGF-containing L-15 media as previously described²⁵. CsA, FK506 and rapamycin were dissolved in 100% ethanol, and applied as 1% volume of the culture media. Control cultures treated with 1% ethanol (vehicle) were always done in parallel. At the indicated times, cultures were observed with differential interference contrast (DIC) microscopy. Real-time imaging throughout showed that growth cones have normal filopodia and confirm the results obtained with replicate neuronal cultures. Three parameters were measured for each condition and time point: the percentage of cells with neurites, the number of neurites per cell, and the lengths of the longest neurites per cell. Multiple ($n > 10$) visual fields were chosen at random from replicate cultures and analysed ($n > 200$ cells in both **a** and **b**). Probability values were calculated by Student's unpaired *t*-test.

activity, such as by activation of the transcription factor NF-ATc⁹.

Next, we tested whether differential calcineurin activity across the growth cone could potentiate guidance decisions and direct growth cone motility. This was done by microscale chromophore-assisted laser inactivation (micro-CALI) of calcineurin in regions of growth cones. CALI is a method of protein ablation that has high spatial and temporal specificity^{10,13}. Localized photochemical damage (half maximal radius = 15 Å) is directed by malachite green-conjugated antibodies to the protein of interest¹², allowing one to inactivate protein function preferentially even within a protein complex¹³. The laser light is not significantly absorbed by other cellular components and does not affect living cells by itself¹⁰. CALI has been used to mimic and dissect known genetic loss of function mutations *in vivo*¹⁴. Using purified proteins *in vitro*, CALI caused a fivefold reduction in calcineurin's phosphatase activity. This inactivation is dependent on both the presence of chromophore-labelled antibodies and on laser irradiation (Fig. 2a). The antiserum used for *in vitro* CALI specifically recognized calcineurin A from chick dorsal root ganglia (DRG) in western blots and in immunocytochemistry (Fig. 2b–d). Next, chromophore-labelled calcineurin antiserum or preimmune serum were loaded into DRG neurons by trituration (Fig. 2e–f). Figure 2g–h shows that CALI of calcineurin within neurons caused a large decrease in the staining of TAU-1 ($P < 0.001$, Student's unpaired *t* test), an antibody specific for the unphosphorylated form of tau, a microtubule-associated protein and known substrate of calcineurin⁴. This demonstrates that CALI inactivates the phosphatase activity of calcineurin within cultured neurons.

Figure 3a–e shows that micro-CALI of calcineurin caused the filopodia and the lamellipodia of the irradiated region to retract and grow around the laser spot. Growth cone behaviour outside

the irradiated area was not affected, and filopodia continued to grow and extend. In contrast, parallel micro-CALI experiments with chromophore-labelled preimmune serum did not perturb growth cone behaviour. The irradiated regions did not retract, and often the filopodia extended into the laser spot (Fig. 3f–j). Quantitative analysis of the average change in filopodial length before and after micro-CALI (Table 1) demonstrated that: (1) calcineurin antiserum alone did not make filopodia retract; (2) there was no systematic bias in the selection of regions of growth cones for micro-CALI; and (3) only local inactivation of calcineurin resulted in significant filopodial retraction (average = 10.6 µm in 10 min). Local retraction was observed in 19 out of 26 (73%) calcineurin micro-CALI experiments but only in 1 out of 19 (5%) micro-CALI experiments with preimmune serum ($P < 0.002$). Filopodial retraction is not a nonspecific trauma response as micro-CALI of talin, an actin binding protein, caused filopodial stalling but not retraction¹⁵. Micro-CALI of calcineurin did not affect neurite extension or the rate of filopodial motility ($P > 0.20$).

Micro-CALI of calcineurin increased the tendency of lateral growth cone movement away from the laser spot by 4.5-fold, and this movement away is significantly associated with filopodial retraction (Table 1). However, we did not observe robust growth cone turning. This may be due to the fact that CALI causes only a temporary loss of function, and growth cone turning may require a continuous asymmetry.

This study presents, to our knowledge, the first experimental evidence that directed filopodial motility can be regulated by the asymmetric inactivation of a protein involved in intracellular signalling. Taken together, the data demonstrate a link from calcineurin inactivation to filopodial retraction and ultimately to lateral movement. The observed response of growth cones to a transient decrease of local calcineurin activity is consistent with

TABLE 1 Summary of micro-CALI data

Change in filopodial length (µm)	Filopodial motility (µm min ⁻¹)			
	Calcineurin antiserum	Preimmune serum	Calcineurin antiserum	Preimmune serum
Before CALI, in laser spot	+0.9 ± 6.0	+2.6 ± 5.5	2.0 ± 1.9	1.2 ± 1.6
After CALI, in laser spot	-10.6 ± 7.8*	+0.7 ± 7.2	1.1 ± 0.9	3.1 ± 3.9
Before CALI, outside laser	+2.9 ± 6.5	-1.3 ± 5.0	3.5 ± 4.4	1.7 ± 1.8
After CALI, outside laser	+0.7 ± 5.3	-0.4 ± 3.3	1.1 ± 1.4	1.6 ± 2.4
Lateral growth cone movement				
<i>n</i>	22	16	Neurite extension (µm)	
Change in movement away	41.00%**	12.5%	αCN before laser	+0.4 ± 0.9
Change in movement toward	9.00%	18.75%	αCN after laser	+1.6 ± 4.5
Odds ratio of moving away	4.5***	0.67	Preimm before	+1.4 ± 2.7
			Preimm after	+0.2 ± 1.6
Regional retraction (number of growth cones)	19/26†	1/19		

Quantitative analysis of micro-CALI data. Data shown are means ± s.d. Average changes in filopodial length, filopodial motility and neurite extension were calculated for the 5 min before and for the 10 min after the start of laser irradiation. For each comparison $n = 10$ growth cones chosen randomly for each condition. When filopodia were compared, two filopodia (chosen randomly, one for each condition) on the same growth cone were used for the 10 growth cones. The large standard deviations are due to the dynamic nature of filopodia and neurites. Filopodial motility (the rate of the change in filopodial length regardless of direction) was calculated by averaging the absolute value of the change in filopodial length every min for the 5 min before and 10 min after the start of laser irradiation.

* $P < 0.001$ by analysis of variance (ANOVA) test and by Student's unpaired *t*-test. The difference among the other seven numbers are not statistically significant ($P > 0.05$). No significant difference was found among all the categories for neurite extension or the rate of filopodial motility ($P > 0.20$ for each, ANOVA tests). Lateral growth cone movement (defined operationally as components of leading edge extension that are perpendicular to the midline of the growth cone) was observed before and after micro-CALI, and changes in lateral movement were scored. 'Moving away' means that the leading edge of the non-laser irradiated side is extending away from the laser spot and does not simply denote lamellipodial retraction.

** Significantly associated with filopodial retraction ($P = 0.03$, McNemar's exact test). Of the growth cones that moved away from the laser spot, 78% also exhibited filopodial retraction.

*** $P < 0.02$ by one sample binomial test, assuming 50% probability for a change in lateral growth cone movement to be away from the laser spot. $P = 0.49$ for the outcome in preimmune micro-CALI experiments.

† $P < 0.002$ by Poisson's test.

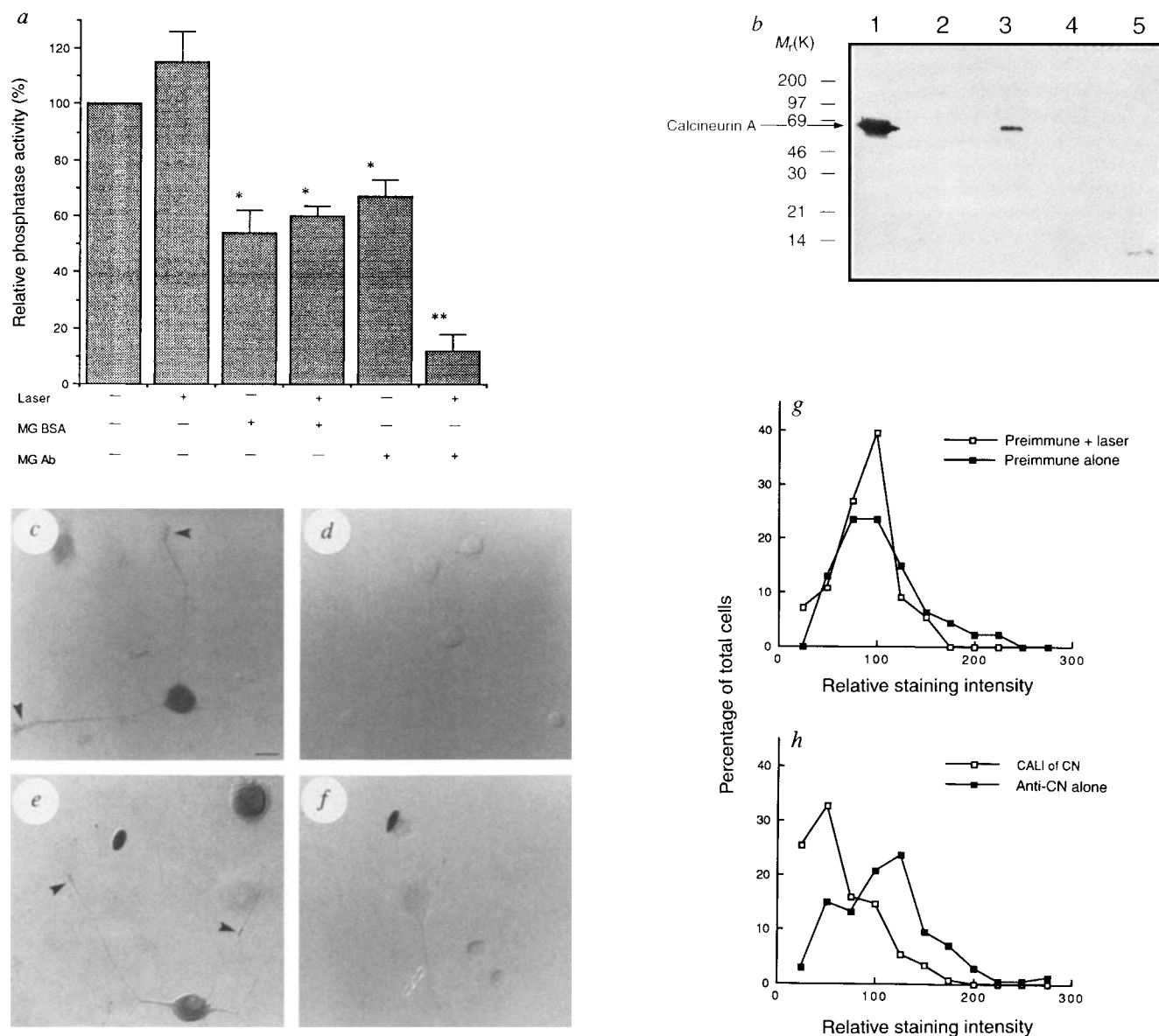


FIG. 2 **a**, CALI of calcineurin (CN) *in vitro* (MG Ab). Dye-labelled protein (such as MG BSA) inhibits CN activity alone, possibly by nonspecific aggregation; CALI with MG BSA causes no additional inactivation. Data shown are mean $\pm$ s.d. ($n=2$). * Significantly different ($P<0.05$) from CN alone or ** from all other values. **b**, Immunoblot loaded with: lanes 1–2, purified bovine CN; lanes 3–5, chick brain extract; lane 5 had 25-fold more protein than lanes 1–4. Lanes 1 and 3 were probed with CN antiserum; 2 and 5 with preimmune serum; 4 with anti-CN preincubated with bovine CN. **c**, Calcineurin A is enriched in neuronal growth cones (arrowheads) and cell bodies, consistent with other studies⁴. Scale bar, 10 μ m. **d**, Immunocytochemistry with preimmune serum shows no staining. **e**, Trituration-loading of anti-calcineurin IgG (90% of the neurons retain the antibody) confirmed by immunocytochemistry with goat anti-rabbit IgG. **f**, Unloaded neurons stained with goat anti-rabbit IgG. **g, h**, Comparison of staining intensity of TAU-1 in DRG neurons subjected to: CALI using preimmune serum (open squares, $n=56$), loading with dye-labelled preimmune serum alone (filled squares, $n=47$), CALI of calcineurin (open squares, $n=150$), and loading with dye-labelled

anti-calcineurin alone (filled squares $n=168$). Neurons were fixed 10 min after laser irradiation.

METHODS. The following were as described: preparation of bovine brain calcineurin and adult chicken brain extract²⁶; IgG purification from rabbit anti-bovine CN antiserum and immunoblotting²⁷; MG labelling and CALI (5 min, 15 mJ, 15:1 MG Ab to CN molar ratio) on purified calcineurin or DRG neurons¹³; PNPP assay of CN²⁸; immunocytochemistry with anti-calcineurin or TAU-1 (ref. 4), an antibody specific for the dephosphorylated form of tau was done as described³⁰. Proteins were loaded into DRG neurons by trituration³⁰. After incubation in 0.25% trypsin at 37 °C for 18 min, DRGs were tritured in Hank's balanced salts saline (Gibco) for **c, d** and **f**, or 3.0 mg ml⁻¹ of anti-calcineurin IgG for **e**. Trituration loading of MG-labelled protein is described in Fig. 3. Pixel density for TAU-1-positive cells was determined using NIH Image software. Relative staining intensity was calculated to normalize and pool data from three different experiments by: (pixel density – background)/(mean pixel density of neurons outside the laser spot).

calcineurin acting as a mediator of localized calcium fluxes. The rapid regional retraction suggests that calcineurin and its effectors must act locally and within minutes to mediate directed filopodial motility.

Although the downstream effectors of calcineurin in the growth cone are not yet known, there are several good candi-

dates. Calcineurin has been reported to dephosphorylate and modulate the activities of GAP-43 (ref. 6), a regulator of growth cone morphology and signalling^{1,16}, tau, a microtubule-associated protein¹⁷, and dynamin I, a protein involved in synaptic vesicle recycling¹⁸. GAP-43 is associated with the cortical F-actin in growth cones¹, and its phosphorylation decreases the growth

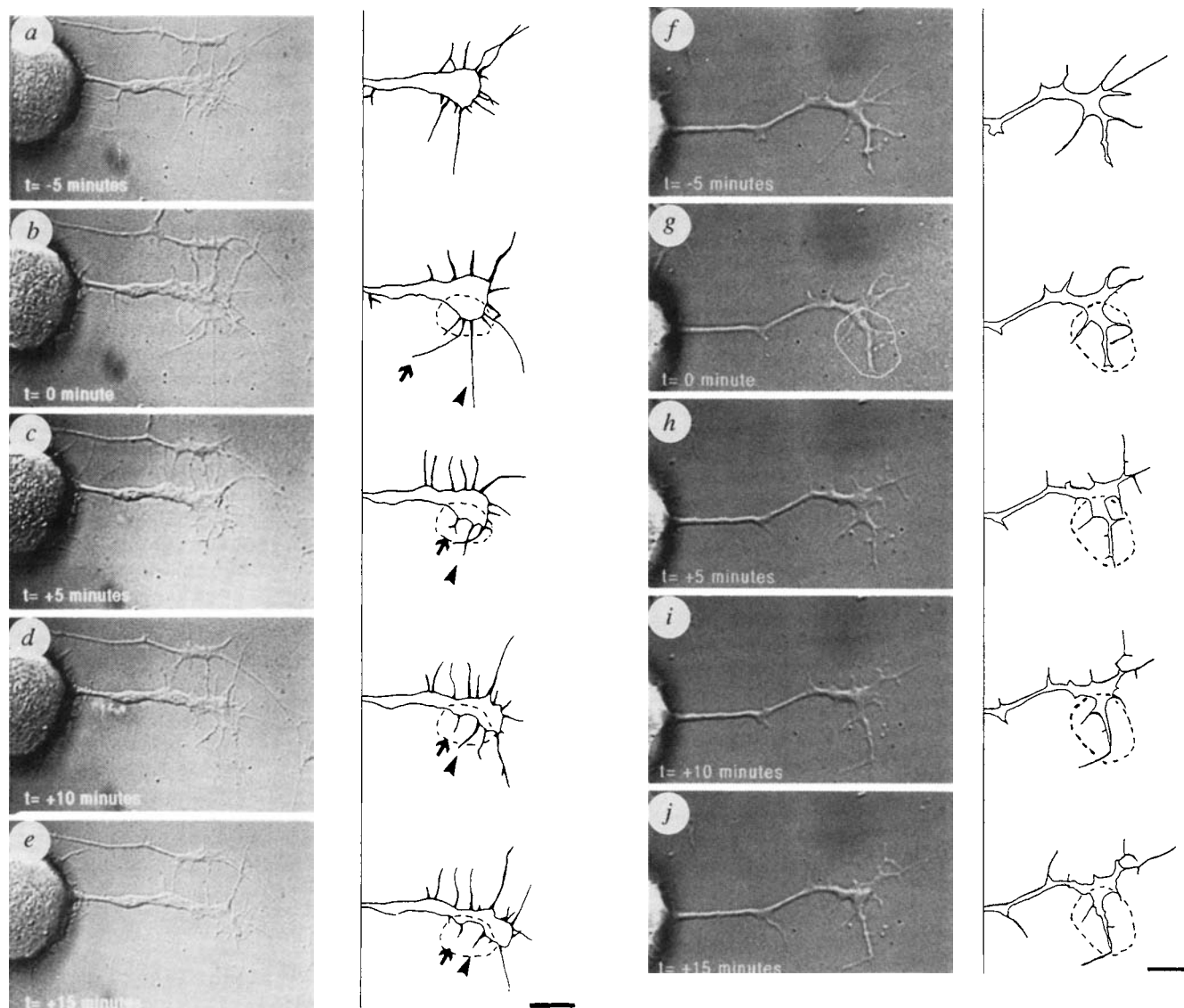


FIG. 3 a–e, Micro-CALI of calcineurin causes regional growth cone retraction. a, A growth cone is first observed for 5 min. b, A region of the growth cone is chosen for micro-CALI (white outline) and laser irradiated for 5 min (t from 0 to +5 min). c, The filopodia and lamellipodia of the irradiated region starts retracting and continues while the rest of the growth cone does not appear to be affected in d and e. e, The irradiated filopodia and lamellipodia have retracted from the laser spot, and the growth cone has grown around the laser spot. The arrow and arrowhead mark two filopodia through the course of the experiment. f–j, Micro-CALI with chromophore-labelled preimmune serum does not perturb growth cone behaviour. A region of the growth cone is chosen for laser irradiation in g, laser irradiated for 5 min ($t=0$ to +5 min), and observed for 10 more minutes in h, i and j. DIC images on left panels; camera lucida drawings on right panels. Scale bar, 10 μm . METHODS. DRG neurons were cultured as described in Fig. 1. Micro-

CALI has been previously described¹¹. The diameter of the laser spot was $\sim 10\ \mu\text{m}$. Malachite green-labelled calcineurin antiserum or pre-immune serum ($3.0\ \text{mg ml}^{-1}$) in Hank's balanced salts saline (Gibco) were loaded into DRG neurons by trituration as described in Fig. 2. To visualize protein loading, fluorescein isothiocyanate conjugated BSA ($1\ \text{mg ml}^{-1}$, final concentration) was included in the protein solution. FITC-positive cells were loaded with antibody as determined by immunocytochemistry. Micro-CALI was performed from 1 to 6 h after plating. Neurons were briefly observed by epifluorescence to verify protein loading. About 75% of the neurons have taken up FITC-BSA and appear fluorescent. DRG neuronal cultures were kept at 37°C with a stage incubator during micro-CALI manipulations. Laser energy for micro-CALI was comparable to that used in large-scale CALI experiments. Growth cone behaviour was observed by time-lapse video microscopy (15 per frame). Image enhancement was aided by custom-written software¹¹.

cone concentration of phosphatidylinositol 4,5-bisphosphate¹⁶, an inhibitor of the actin-severing protein gelsolin¹⁹. Thus local inhibition of calcineurin may increase the severing of actin filaments and lead to filopodial retraction.

Filopodia play key roles in signal detection²⁰ and motile force generation²¹, and preferential filopodial sprouting has been implicated in growth cone guidance²². Thus directed filopodial retraction in response to local deficiencies of calcineurin activity may be an important mechanism for growth cone guidance *in vivo*. Calcineurin has been implicated in neutrophil

chemokinesis²³. We speculate that asymmetric calcineurin activity may be a general mechanism for translating spatially distributed extracellular cues into directed cellular motility. □

Received 10 May; accepted 20 June 1995.

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ACKNOWLEDGEMENTS. We thank C. T. Walsh and K. Nadeau for CsA and for discussion, S. L. Schreiber and M. Albers for FK506 and rapamycin, and F. D. McKeon, A. Lander and S. L. Schreiber for critical review of the manuscript. This work was supported by grants from the NIH, the Lucille P. Markey Charitable Trust and the Ford Program. D.G.J. is a Klingenstein Fellow and John L. Loeb Associate Professor of the Natural Sciences.

A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem

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POTASSIUM channels catalyse the permeation of K⁺ ions across cellular membranes and are identified by a common structural motif, a highly conserved signature sequence of eight amino acids in the P domain of each channel's pore-forming α -subunit^{1,2}. Here we describe a novel K⁺ channel (TOK1) from *Saccharomyces cerevisiae* that contains two P domains within one continuous polypeptide. *Xenopus laevis* oocytes expressing the channel exhibit a unique, outwardly rectifying, K⁺-selective current. The channel is permeable to outward flow of ions at membrane potentials above the K⁺ equilibrium potential; its conduction-voltage relationship is thus sensitive to extracellular K⁺ ion concentration. In excised membrane patches, external divalent cations block the channel in a voltage-dependent manner, and their removal in this configuration allows inward channel current. These attributes are similar to those described for inwardly rectifying K⁺ channels^{3,4}, but in the opposite direction, a previously unrecognized channel behaviour. Our results identify a new class of K⁺ channel which is distinctive in both its primary structure and functional properties. Structural homologues of the channel are present in the genome of *Caenorhabditis elegans*.

The peptide sequence of the P domain from several K⁺ channel proteins was used to search gene databases stored at the National Center for Biotechnology Information (NCBI) using

the BLAST sequence alignment program⁵. Along with many known K⁺ channel genes, one cosmid from the *S. cerevisiae* genome sequencing project (chromosome X) was identified⁶. Translation of one extended open reading frame (ORF J0911) reveals a hypothetical protein with two P-like domains, each containing an octapeptide similar to the K⁺ channel signature sequence (Fig. 1a). Hydropathy analysis shows that both P-like domains are bounded by hydrophobic segments and distinguishes eight potential transmembrane helices (Fig. 1b). Marked sequence homology is apparent between the hydrophobic segments following each P-like domain and the S6 domains of known K⁺ channels (Fig. 1c). The gene was isolated from yeast genomic DNA by polymerase chain reaction (PCR). Northern blot analysis confirmed that a transcript of 2.2 kilobases (kb), the expected size for ORF J0911, is present in total yeast RNA and reverse transcription (RT)-PCR was used to delineate the 3' end of the transcript (Fig. 1, methods). Complementary RNA was transcribed from this putative yeast K⁺ channel gene (TOK1) and injected into *Xenopus laevis* oocytes.

One day after cRNA injection, an outwardly rectifying and non-inactivating current, not present in uninjected cells, was measured by two-electrode voltage clamp (Fig. 2a). Unlike voltage-gated K⁺ channels, which are activated by membrane depolarization over a fixed voltage range to pass outward current, the voltage at which TOK1-induced currents are first observed changes in response to external K⁺ ion concentration (Fig. 2b). The activation potentials of TOK1 currents, defined as the voltage where the slope conductance increases noticeably⁷, are -94 ± 4 mV in 2 mM KCl, -46 ± 6 mV in 20 mM KCl, and -2 ± 4 mV in 100 mM KCl ($n = 10$ oocytes; Fig. 2), close to the K⁺ equilibrium potential (E_K) predicted for each solution by the Nernst equation. These changes in activation potential are also apparent in normalized conductance-voltage plots (Fig. 2c), indicating that shifts cannot be attributed to driving force-induced changes in TOK1 current magnitude. That only minimal inward currents are observed with 20 or 100 mM external KCl at negative voltages (even though there is outward current in 2 mM KCl) supports the premise that the effect of voltage is not primarily intrinsic to TOK1 (Fig. 2b).

Whole-cell TOK1 currents are selective for K⁺ over both Na⁺ and Cl⁻. The magnitude of TOK1 currents and the voltage at which they develop are insensitive to substitution of chloride by aspartate (Fig. 2d, $n = 6$) and to substitution of sodium by N-methylglucamine ($n = 5$; Fig. 2, methods). Reversal potentials under pseudo-bi-ionic conditions⁸ indicate a permeability ratio (P_K/P_{Na}) of at least 20 (Fig. 2e). TOK1 currents are inhibited by barium and tetraethylammonium with inhibition constants at 0 mV of 0.06 ± 0.01 mM and 5.7 ± 0.7 mM, respectively ($n = 5$, 2 mM KCl solution at 10 ms) but are insensitive to 200 nM charybdotoxin, 150 nM iberiotoxin and 200 nM dendrotoxin ($n = 2$, 2 mM KCl solution). These results suggest that cRNA injection leads to expression of a K⁺-selective channel that passes outward current at membrane potentials more positive than E_K .

On-cell patch recording confirms that TOK1 currents are mediated by ion channels (Fig. 3). The channels are not seen in patches from uninjected oocytes ($n = 8$ cells, 14 patches) or oocytes injected with three other K⁺ channel cRNAs (minK, $n = 4$ cells, 16 patches; dSLO, $n = 10$ cells, 30 patches; mSLO, $n = 55$ cells, >150 patches)^{9–11} but are observed in all oocytes injected with 1–5 ng TOK1 cRNA ($n = 140$ cells, >350 patches). TOK1 channels in on-cell patches pass outward current in response to depolarization (Fig. 3a). Channel behaviour appears largely unaltered by patch excision (Fig. 3b); channels do not 'run down' and display no apparent requirement for intracellular constituents. Like whole-cell currents, single channel activity is seen to shift with changes in E_K (Fig. 3c). That TOK1 channels are selective for K⁺ over Na⁺ is confirmed by elimination of channel activity when NaCl is isotonically substituted for internal KCl in the solution bathing inside-out patches (Fig. 3d, $n = 4$). Single TOK1 channels have an apparent conductance of

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a

1 MTRFMNSFAK QTLGYGNMAT VEQESSAQAV DSHSNVTPKQ AKGVLAELK

S1

51 DALRFRDERV SIINAEPSS LFVFEVVS YFPVITACLG PVANTISIAC

S2

101 VVEKWRSLKN NSVVTNPRSN DTDVLMNQVK TVFDPPGIFA VNIISLVGLF

S3

151 TSNIILMLHF SKKLTLYLKSQ LINITGWTTA GGMLLVQVIV CSLNDMFSIY

S4

201 SKTIGFWFAC ISSGLYLVT IILTIFIGY KLKYPPTFN LLPNERSIMA

S5 **P₁**

251 YTVLLSLWLI WGAGMFSGLL HITYGNALYF CTVSLITVGL GDILPKSVGA

S6

301 KIMVLIFSLS GVVLMGLIVF MRSIIQKSS GPIFFHHRVE KGRSKSWKHV

S7

351 MDSSKNLSER EAFDLMKICR QTASRKQHWF SLSVTIAIFM AFWLLGALVF

P₂ **S8**

401 KFAENWSYFN CIYFCFLCLL TIGYGDYAP TGAGRAFFVI WALGAVPLMG

451 AILSTVGDLL FDISTSLDIK IGESFNKVK SIVENGRQRA LSFVMTGEI

501 FEESDTADGD LEENTSSQS SQISEFNDNN SEENDSGVTS PPASLQESFS

551 SLSKASSPEG ILPLEYVSSA EYALQDSGTC NLRNLQELLK AVKKLHRIEL

601 ADKDYTLFS DWSYIHLHL RNITDIEEYT RGPEFWISPD TPLKFLNPEP

651 HFAFMMLFKN IEELVGNLVE DEELYKVISK RKFLGHRKT L

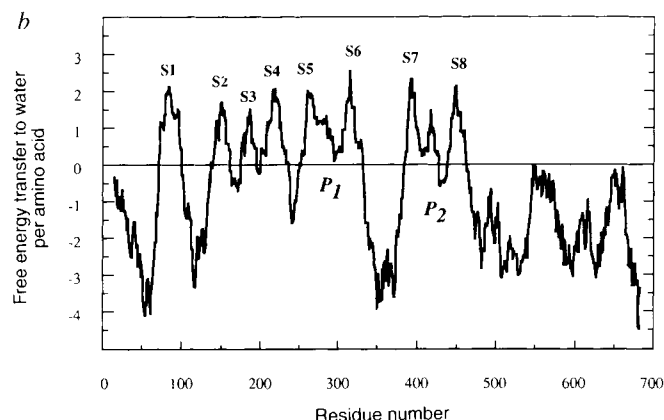


FIG. 1 Primary structure of the TOK1 protein, its hydropathy profile and alignment with related sequences. **a**, The predicted protein sequence of yeast ORF J0911 (accession #X77087). Segments homologous to the *P* domains of known K⁺ channels are bold, and residues corresponding to the K⁺ channel signature sequence (TXXTXGYG)² are double underlined. Hydrophobic segments are labelled S1–S8. **b**, Hydropathy plot of the predicted protein sequence. Average free energy of transfer to water was calculated per amino acid with a window of 20 amino acids²¹. Eight potential transmembrane helices and the two *P* domains are designated. **c**, Alignment of each TOK1 *P* domain and its flanking residues with known K⁺ channels and a predicted ORF from *C. elegans* (*Cel*). Residues identical or similar to TOK1 are bold. BLAST analysis shows the P₁–S6 regions of TOK1 to be 40% identical and 70% similar to the P–S6 regions of the mammalian voltage-gated K⁺ channel Kv4.2 (#M59980), and the S7–P₂–S8 regions of TOK1 to be 32% identical and 50% similar to the S5–P–S6 regions of the *Drosophila* Ca²⁺-activated K⁺ channel dSlo (#M96840). Hypothetical ORFs from *C. elegans* which predict proteins containing two *P* domains are F22B7.7 (#L12018) and F17C8.5 (#Z35719). The predicted

c

Pre-pore regions

TOK1	S5	V L L S L W L I W G A G M F S G L L H I T Y
TOK1	S7	I F M A F W L L . G A L V F K F A E N W S Y
<i>Cel</i>	pre-P ₁	F E K Y F L T S N E V K K N A A T E T W T F
<i>Cel</i>	pre-P ₂	A I L I V Y T A F G G V L M S K L E P W S F
Kv4.2	S5	I I F A T V M F Y A E K G S S A S K F T S I
dSlo	S5	I I H L L E N S G D P L D F D N A H R L S Y

Pore domains

		P₁
TOK1	P ₁	G N A L Y F C T V S L L T V G L G D I L P
<i>Cel</i>	P ₁	S S S I F F A V T V V T T I G Y G N P V P
Kv4.2	P	P A A F W Y T I V T M T T L G Y G D M V P

		P₂
TOK1	P ₂	F N C I Y F C F L C L L T I G Y G D Y A P
<i>Cel</i>	P ₂	F T S F Y W S F I T M T T V G F G D L M P
dSlo	P	W T C V Y F L I V T M S T V G Y G D V Y C

Post-pore regions

TOK1	S6	K S V G A K I M V L I F S L S G . . V V L
TOK1	S8	R T G A G R A F F V I W A L . . G A V P L
<i>Cel</i>	post-P ₁	V T N I G R I W C I L F S L L G I P L T L
<i>Cel</i>	post-P ₂	R R D G Y M Y I I L L Y . I I L G K F S M
Kv4.2	S6	K T I A G K I F G S I C S L S G V L V I A
dSlo	S6	E T V L G R T F L V F F L L V G L A M F A

sequence of F22B7.7 (*Cel*) has a hydropathy profile similar to TOK1 from S4 to S8 and exhibits 20% identity and 52% sequence similarity over this region. The probabilities that six random sequences would have the amino acid identities shown here are 1×10^{-17} for the *P* domains, and 2×10^{-5} for the post-*P* domain regions by the method of Jan and Jan²²; homology is not significant in the pre-*P* domain regions although marked homology is evident between TOK1 S5 and S7. The TOK1 encoding portion of ORF J0911 has been redesignated by the accession number U28005.

METHODS. Default parameters were used in the BLAST search which identified ORF J0911. Hydropathy analysis and sequence alignments were performed using GCG software (Genetics Computer Group, Inc.). ORF J0911 was amplified by PCR from yeast genomic DNA using primers corresponding to nucleotides 3027–3055 and 5101–5121 of cosmid clone 233 (numerical designations correspond to the published sequence and refer to the complementary DNA strand)⁶. Additional nucleotides were included at the 5' end of each primer to construct the restriction sites *Sall*, *Bam*HI, and *Bgl*III, *Hind*III on the 5' and 3' ends of the gene, respectively (5'-GCCACCGAAGCTTAGATCTGCTCGGGTG-TTTGTATATCAAGTGTC-3' and 5'-GCCACCGGTCGACGGATCCATGACAA-GGTTTCATGAACAGC-3'). The amplified fragment was digested with *Sall* and *Hind*III and cloned into pBluescript (Stratagene). This construct was sequenced in its entirety and shown to be identical to the published nucleotide sequence of ORF J0911 (ref. 6). Northern blots of total yeast RNA were probed with ³²P-labelled cRNA prepared to the complementary DNA strand. 3' RACE (rapid amplification of cDNA ends; GIBCO BRL) from total yeast RNA located the poly(A) tail at nucleotide 2917, 3' to the putative transcription termination/polyadenylation signal previously identified at position 2981 (ref. 6). Whether there are multiple initiation sites for this gene is not yet known. For production of cRNA the gene was transferred to pGEM-A²³; this construct is identified as pTOK1.

roughly 35 pS in symmetrical K^+ , and a 'flickery' appearance under these recording conditions (Fig. 3e).

The current-voltage (I - V) relationship of inward rectifier K^+ channels results from the blocking of outward current by internal Mg^{2+} and polyamines at voltages above E_K (refs 3, 4, 12-15).

Removing divalent cations from the external channel face allows inward flux of K^+ ions through TOK1 channels in off-cell patches at hyperpolarized potentials that were previously electrically quiet (Fig. 4a, $n=3$ cells, 5 patches). Removing external divalent cations also results in more outward TOK1 current

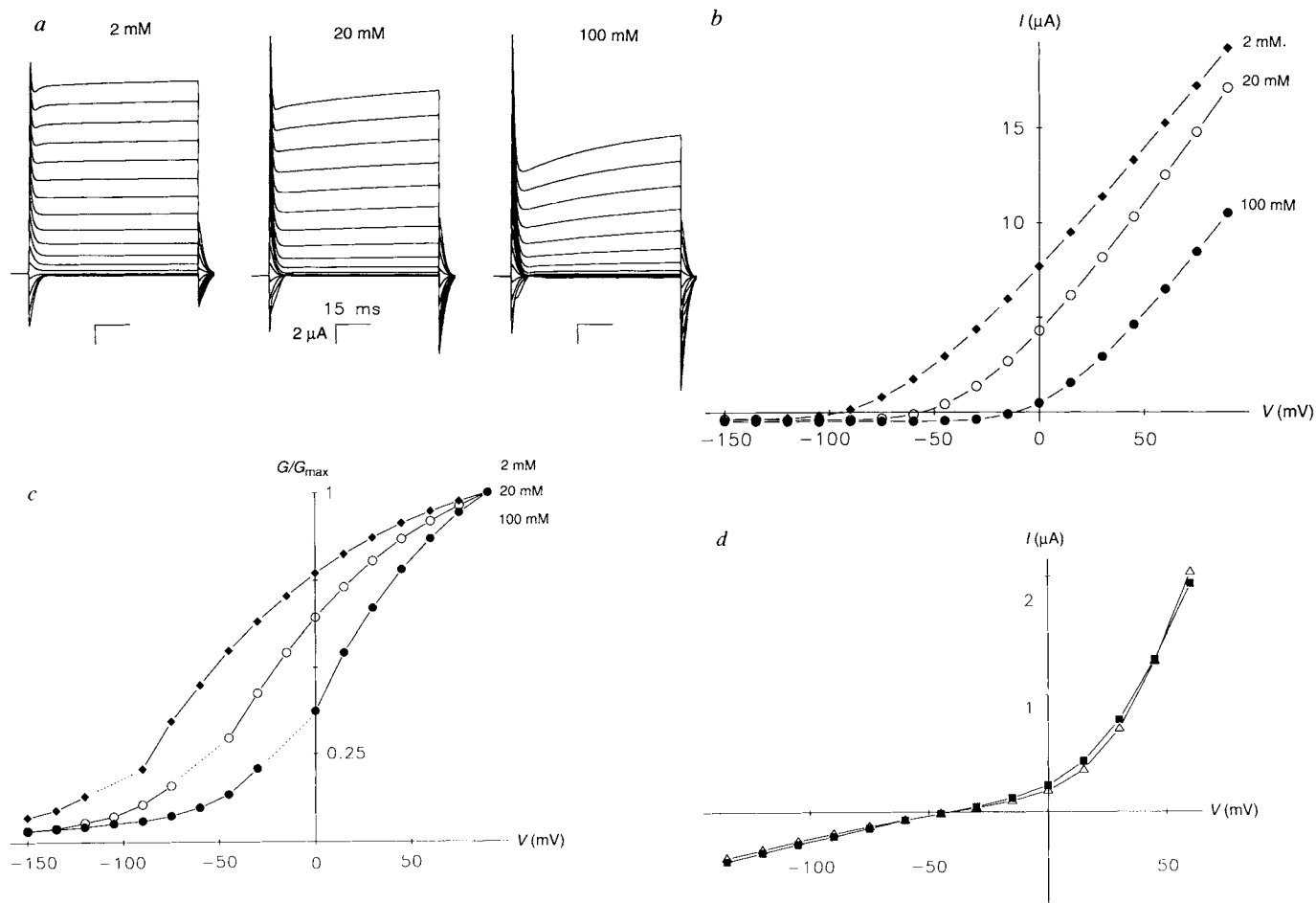


FIG. 2 TOK1-induced currents in *X. laevis* oocytes assessed by two-electrode voltage clamp show outward rectification and activation potentials that shift with external KCl concentration. **a**, Currents elicited by 75 ms pulses from -150 to +90 mV in 15 mV steps from a holding potential of -80 mV with a 1-s interpulse interval and displayed without leak subtraction; bath solution was 2 mM KCl solution, then 20 mM KCl solution, and then 100 mM KCl solution. Scale bars, 2 μ A and 15 ms. **b**, Current-voltage relationship at 10 ms into the test pulses shown in **a**; 2 mM KCl solution (filled diamonds), 20 mM KCl solution (open circles) and 100 mM KCl solution (filled circles). **c**, Normalized conductance-voltage relationship at 10 ms into the test pulses shown in **a**; 2 mM KCl solution (filled diamonds), 20 mM KCl solution (open circles) and 100 mM KCl solution (filled circles); E_K was taken as the reversal potential **a**. **d**, Currents at 250 ms otherwise as in **a** in 98 mM KCl solution (open triangles) and then 98 mM KAspartate solution (filled squares). **e**, Currents at 10 ms elicited as in **a** in 100 mM KCl solution (filled squares) and 100 mM NaCl solution (open triangles); the inset shows data points near the reversal potential on an expanded scale. **METHODS**. cRNA was prepared after linearization of pTOK1 by NotI and transcription with T7 polymerase as described²⁴. Transcript concentration was estimated spectrophotometrically and portions stored at -80 °C. *X. laevis* oocytes were isolated and injected with 46 nl cRNA solution containing 1-5 ng transcript as described previously²⁴. Whole oocyte currents were recorded 1-4 days after cRNA injection using a two-electrode voltage clamp (Oocyte Clamp, Warner Instruments). Electrodes contained 3 M KCl and had resistances of 0.3-1.0 M Ω . Recordings were performed under constant perfusion at room temperature. Bath solutions were: 2 mM KCl solution (containing in mM: 98 NaCl, 2 KCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES, pH 7.6); 20 mM KCl solution (in mM: 80 NaCl, 20 KCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES, pH 7.6); 100 mM KCl solution (in mM: 100 KCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES, pH 7.6); 98 mM KCl solution (in mM: 98 KCl, 0.3 CaCl₂, 5 HEPES,

pH 7.6); 98 mM KAspartate solution (in mM: 98 KAspartate, 0.3 CaOH₂, 5 HEPES, pH 7.6); 100 mM NaCl solution (in mM: 100 NaCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES, pH 7.6); and 2 mM KCl/98 mM *N*-methylglucamine (NMG) solution (in mM: 98 NMG, 2 KCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES, pH 7.6).

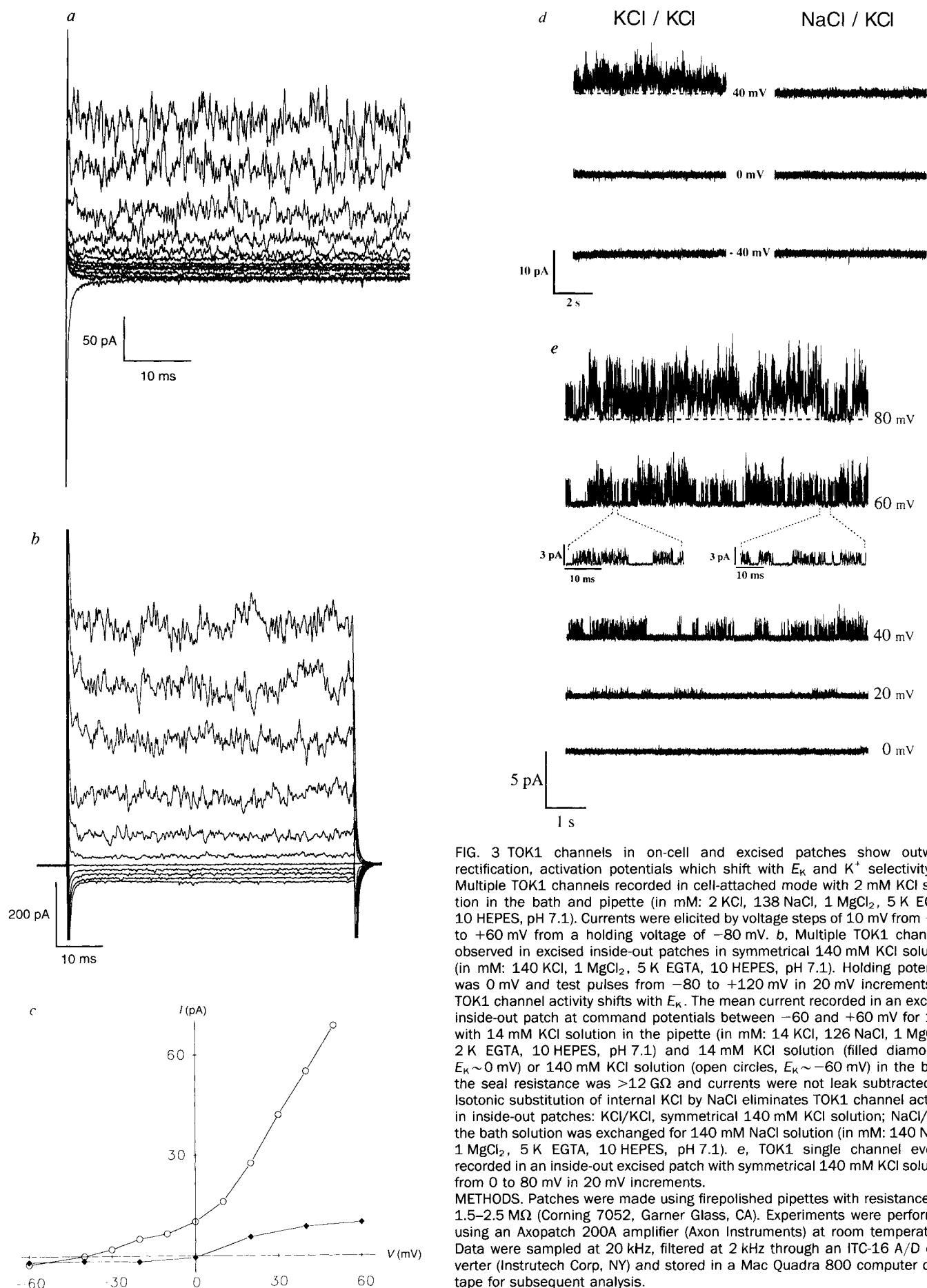


FIG. 3 TOK1 channels in on-cell and excised patches show outward rectification, activation potentials which shift with E_K and K^+ selectivity. **a**, Multiple TOK1 channels recorded in cell-attached mode with 2 mM KCl solution in the bath and pipette (in mM: 2 KCl, 138 NaCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1). Currents were elicited by voltage steps of 10 mV from -60 to +60 mV from a holding voltage of -80 mV. **b**, Multiple TOK1 channels observed in excised inside-out patches in symmetrical 140 mM KCl solution (in mM: 140 KCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1). Holding potential was 0 mV and test pulses from -80 to +120 mV in 20 mV increments. **c**, TOK1 channel activity shifts with E_K . The mean current recorded in an excised inside-out patch at command potentials between -60 and +60 mV for 10 s with 14 mM KCl solution in the pipette (in mM: 14 KCl, 126 NaCl, 1 $MgCl_2$, 2 K EGTA, 10 HEPES, pH 7.1) and 14 mM KCl solution (filled diamonds, $E_K \sim 0$ mV) or 140 mM KCl solution (open circles, $E_K \sim -60$ mV) in the bath; the seal resistance was >12 G Ω and currents were not leak subtracted. **d**, Isotonic substitution of internal KCl by NaCl eliminates TOK1 channel activity in inside-out patches: KCl/KCl, symmetrical 140 mM KCl solution; NaCl/KCl, the bath solution was exchanged for 140 mM NaCl solution (in mM: 140 NaCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1). **e**, TOK1 single channel events recorded in an inside-out excised patch with symmetrical 140 mM KCl solution from 0 to 80 mV in 20 mV increments.

METHODS. Patches were made using firepolished pipettes with resistances of 1.5–2.5 M Ω (Corning 7052, Garner Glass, CA). Experiments were performed using an Axopatch 200A amplifier (Axon Instruments) at room temperature. Data were sampled at 20 kHz, filtered at 2 kHz through an ITC-16 A/D converter (Instrutech Corp, NY) and stored in a Mac Quadra 800 computer or to tape for subsequent analysis.

(Fig. 4a). This leads to a roughly linear I - V relationship from -90 to $+90$ mV, even in the presence of Mg^{2+} at the intracellular patch surface (Fig. 4b). Returning divalent cations to the external bath solution eliminates inward currents at hyperpolarized potentials, thus restoring outward rectification (Fig. 4). These results support the notion that voltage-dependent blocking events contribute to outward rectification of TOK1 channel currents. That TOK1 I - V relationships in excised patches (Fig. 3c) and whole cells (Fig. 2) shift with E_K is also consistent with this hypothesis. However, that divalent cations block inward TOK1 currents in excised membrane patches does not indicate that they are the only, or even the primary, mediators of rectification *in vivo*.

These data do not support the idea that ion channels endogenous to oocytes mediate TOK1 currents. TOK1 channels are not observed in uninjected oocytes, nor in oocytes injected with other channel cRNAs, even in the absence of external divalent cations ($n=9$; Fig. 4c). The channel characterized here is observed only in patches from oocytes injected with TOK1 cRNA. Two endogenous channels^{16,17} can be seen in patches from both control and TOK1 cRNA-injected cells but are clearly different from TOK1 channels. The first has a small conductance (20–40 pS), similar to TOK1 channels, but is insensitive to substitution of KCl by NaCl ($n=2$), and is observed only at membrane potentials of 120 mV and above. The second has a very large conductance (>350 pS), is activated by large potentials (± 120 mV), and thereafter is observed at all voltages.

A notable kinetic difference is seen between TOK1 whole-cell and patch currents. Whole-cell currents have an initial rapid phase followed by a slower second component that is apparent with high external KCl and at higher potentials (Fig. 2a). Patch currents show no time-dependent phase (Fig. 3a, b). A similar phenomenon in *eag* K^+ channels was explained by Ca^{2+} -activated chloride channel activity¹⁸, but the slow component seen with TOK1 is not altered by substitution of chloride with aspartate (Fig. 2d) or by removal of all Ca^{2+} from bath solutions ($n=16$). Slow-phase current magnitude in TOK1 increases with external K^+ concentration (Fig. 2a) and appears to reflect a process specific to K^+ ions.

There are two unusual features of TOK1 channels. First, TOK1 appears to be the first member of a new K^+ channel family defined structurally. In contrast to previously identified K^+ channel α -subunits, which bear only one pore-forming P region, TOK1 has two. A search of the NCBI database with the TOK1 coding region using the BLAST algorithm identified two hypothetical proteins in the *C. elegans* genome on chromosome III which also have a predicted structure with two P domains (Fig. 1c). Second, TOK1 appears to be the first example of a new functional type of K^+ -selective ion channel, an 'outward rectifier' which, by analogy to the inward rectifier superfamily, passes outward K^+ current with an activation potential that is coupled to E_K . Our findings suggest that the mechanism of rectification may be similar in the two families, that is, voltage-dependent blockade. It is premature to conclude that all channels with this type of outward rectifier behaviour will contain two P domains in tandem, or that a structure with two P domains will herald only an outward rectifier K^+ channel phenotype.

Four α -subunits containing P regions associate to form one pore in *Shaker*-type channels^{19,20}. This arrangement resembles the structural organization of voltage-dependent Na^+ and Ca^{2+} channel α -subunits in which four homologous domains are repeated, each contributing one P -like region. The presence of two P domains in TOK1 suggests that it may function as a dimer. Equally provocative is the hydropathy analysis of TOK1, which is crudely reminiscent of a *Shaker*-like channel (with P_1 placed between S5 and S6) attached to an inward rectifier-like channel (with P_2 positioned between S7 and S8) (Fig. 1b). Protein biochemistry will be required to evaluate the stoichiometry and membrane topology of TOK1 channels. In conclusion, ORF J0911 from *S. cerevisiae* chromosome X encodes a functional

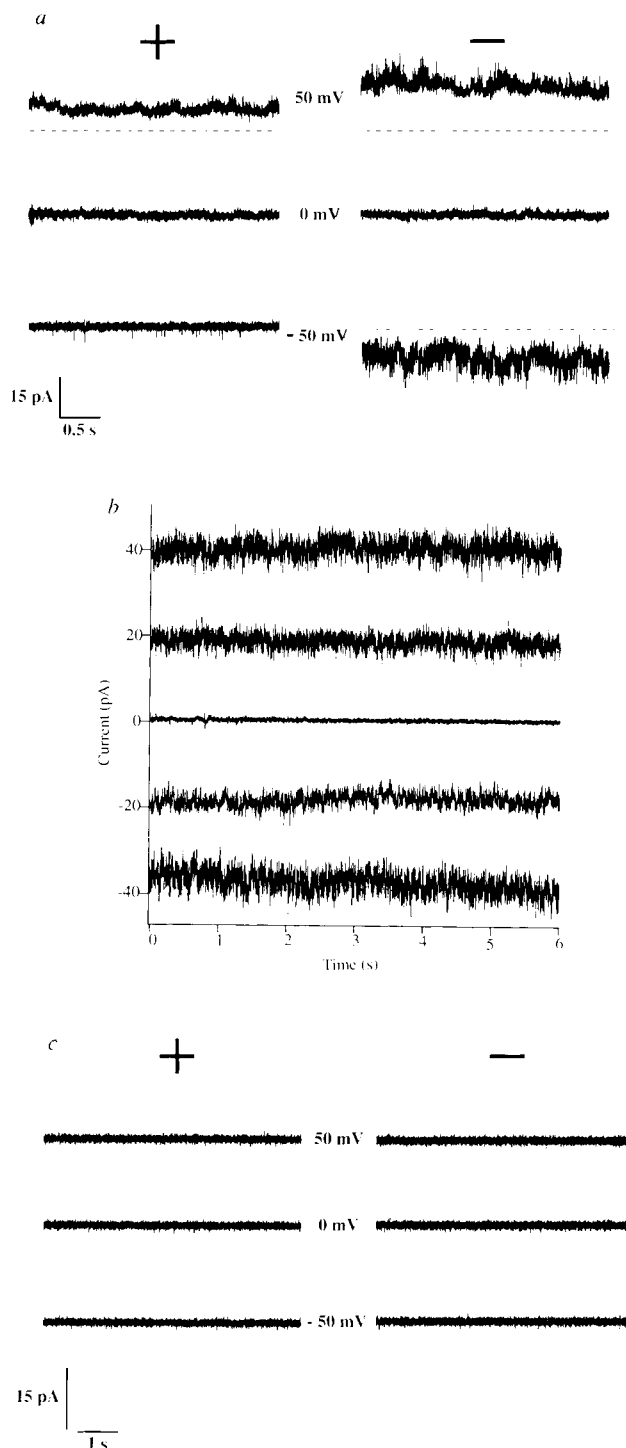


FIG. 4 Divalent-free solution at the external channel face allows inward TOK1 currents in excised membrane patches. **a**, TOK1 channels recorded in the same excised outside-out patch with 140 mM KCl solution and divalent cations in the bath (+; in mM: 140 KCl, 1 Mg^{2+} , 0.3 Ca^{2+} , 10 HEPES, pH 7.1) and after the bath solution was exchanged for 140 mM KCl nominally divalent-free solution (-; in mM: 140 KCl, 2 K EGTA, 2 K EDTA, 10 HEPES, pH 7.1); the pipette solution was 140 mM KCl solution (in mM: 140 KCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1); holding voltages were +50, 0 and -50 mV. Repetitive exchange of solutions bathing these patches showed effects to be fully reversible. **b**, TOK1 channels recorded in an excised inside-out patch with 140 mM KCl nominally divalent-free solution in the pipette and 140 mM KCl solution in the bath at +90, 50, 0, -50 and -90 mV. **c**, Excised outside-out patches from oocytes injected with 2 ng minK cRNA show no current under divalent-free conditions identical to **a**. Data were sampled as in Fig. 3.

protein with two *P* domains that behaves as an outward rectifier K^+ channel whose activation is coupled to E_K ; it has been given the name TOKI. □

Received 7 April; accepted 26 June 1995.

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ACKNOWLEDGEMENTS. A.J.S. thanks F. Sigworth for guidance in the development and use of patch-clamp analysis computer software. K.A.K. thanks C. W. Slayman for advice, financial support and encouragement. We thank C. Miller and F. Sigworth for reviews of the manuscript, K. Allen and J. Du for technical assistance, and A. M. Quinn for assistance with sequence analyses. This work was supported by the NIH. K.A.K., A.J.S. and S.A.N.G. are members of the Molecular Cardiology Program of the Yale University Boyer Center for Molecular Medicine; the Program is supported in part by a grant from American Home Products—Lederle Laboratories. W.J.J. is supported by a predoctoral fellowship from the Howard Hughes Medical Institute.

Identification of a mouse male-specific transplantation antigen, H-Y

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THE male-specific transplantation antigen, H-Y, causes rejection of male tissue grafts by genotypically identical female mice¹ and contributes to the rejection of human leukocyte antigen-matched male organ grafts by human females². Although first recognized 40 years ago³, the identity of H-Y has remained elusive. T cells detect several distinct H-Y epitopes^{3–5}, and these are probably peptides, derived from intracellular proteins, that are presented at the cell surface with major histocompatibility complex (MHC) molecules⁶. In the mouse, the gene(s) controlling H-Y expression (*Hya*) are located on the short arm of the Y chromosome^{7,8} between the zinc-finger genes *Zfy-1* and *Zfy-2* (ref. 9). We have recently identified *Smcy*, a ubiquitously expressed gene, in this region¹⁰ and its X-chromosome homologue, *Smcx*¹¹. Here we report that *Smcy*

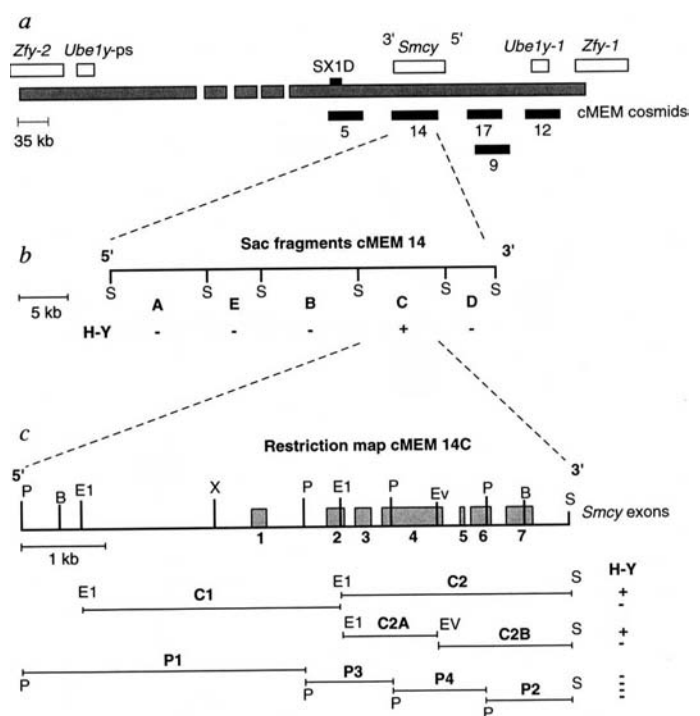


FIG. 1 Localization of the H-YK^k epitope to part of the *Smcy* gene. *a*, The organization of the ΔSxr^b deletion interval of the short arm of the mouse Y chromosome. The extent of the ΔSxr^b deletion interval is indicated by the thick grey line. The positions of genes *Zfy-1*, *Zfy-2*, *Smcy*, *Ube1y* and a partial pseudogene of *Ube1y* are indicated by open boxes. The position of the Y-specific anonymous marker, *Sx1D*, is shown as the filled box above the line, and the positions of the cosmids by the filled boxes below the line. cMEM 12 contains the entire *Ube1y* gene. cMEM 14 contains the 3' end and ~5 kb of *Smcy* exons. cMEMs 5, 9 and 17 do not contain any known genes. *b*, Subcloning of cMEM 14. The cosmid was subcloned into five *Sac*I fragments (cMEM 14A-E) which included all but 1.5 kb of the cosmid DNA. The order of these subfragments was established by hybridization with cDNAs of different lengths (data not shown). The H-YK^k typing of cells transfected with these fragments is indicated by + (positive) and – (negative). *c*, Restriction site and exon mapping of cMEM 14C. A restriction map was constructed with *Bam*HI (B), *Eco*RI (E1), *Eco*RV (EV), *Pst*I (P) and *Xho*I (X). Exons were positioned by comparison of genomic sequence with the sequence of the human X homologue, *XE169*, cDNA²⁶ and a *Smcy* cDNA. The H-YK^k typing results for the subclones MEM 14C1, C2, C2A, C2B and the *Pst*I fragments are indicated by + or –.

METHODS. Cosmids cMEM 5, 9, 12, 14 and 17 were derived from a cosmid contig isolated from an *XY*^{Sxr^b cosmid library and a C3H YAC clone covering this region, and are of C3H origin. Subclones were all inserted into pBluescript II KS+ (Stratagene) except cMEM 14A, which was subcloned by recircularizing the cosmid vector pWE15 after *Sac*I digestion. Fragment C was mapped by indirect labelling of partial digests, and by complete digests. Sequencing was performed as described¹⁴ using subclones and exonuclease III/mung bean nuclease generated nested deletions.}

encodes an H-YK^k epitope that is defined by the octamer peptide TENGSKDI: no similar peptide is found in *Smcx*. These findings provide a genetic basis for the antigenic difference between males and females that contributes towards a tissue transplant rejection response.

The genes controlling H-Y expression, *Hya*^{7,9,12}, spermatogenesis, *Spy*¹³, and the ubiquitin-activating enzyme homologue, *Ube1y*^{14,15}, are located on the short arm of the mouse Y chromosome between the zinc-finger genes, *Zfy-1* and *Zfy-2*, in the ΔSxr^b deletion interval (Fig. 1a). To identify *Hya* we adopted a cosmid transfection approach^{16,17}. Five cosmids from this region were tested for their ability to direct H-Y expression after transfection into female PIHtr cells expressing H-2K^k (P1K^k). P1K^k

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cells transfected with four of the cosmids (cMEMs 5, 9, 12 and 17) failed to stimulate either an H-YK^k-specific T-cell clone, C6 (ref. 18) or 2A11, a hybridoma derived from C6 (ref. 12). This is shown in Fig. 2a for the cosmid cMEM 12, which contains the entire *Ubel* gene. The transfectants were tested for *Ubel* expression by reverse transcription polymerase chain reaction (RT-PCR) and two of three were positive (data not shown). The remaining cosmid, cMEM 14, contains most of the *Smcy* gene. Cells transfected with cMEM 14 consistently stimulated both the T-cell clone, C6, and the hybridoma, 2A11 (Fig. 2b),

indicating that *Smcy* encodes an H-YK^k epitope. The transfectants expressing H-YK^k did not express the H-YA^b epitope, as determined by a previously described assay¹⁹ (data not shown).

To define further this H-YK^k epitope, DNA restriction fragments from cMEM 14 were subcloned (Fig. 1b), transfected into P1K^k cells and the cells analysed for H-Y expression. Only those transfected with a *Sac*I-digested DNA fragment, cMEM 14C, expressed H-YK^k (Fig. 2c). This fragment contains the seven most 3' exons of *Smcy* (designated 1–7; Fig. 1c). Transfection of cloned restriction fragments of cMEM 14C (Fig. 2d, e) local-

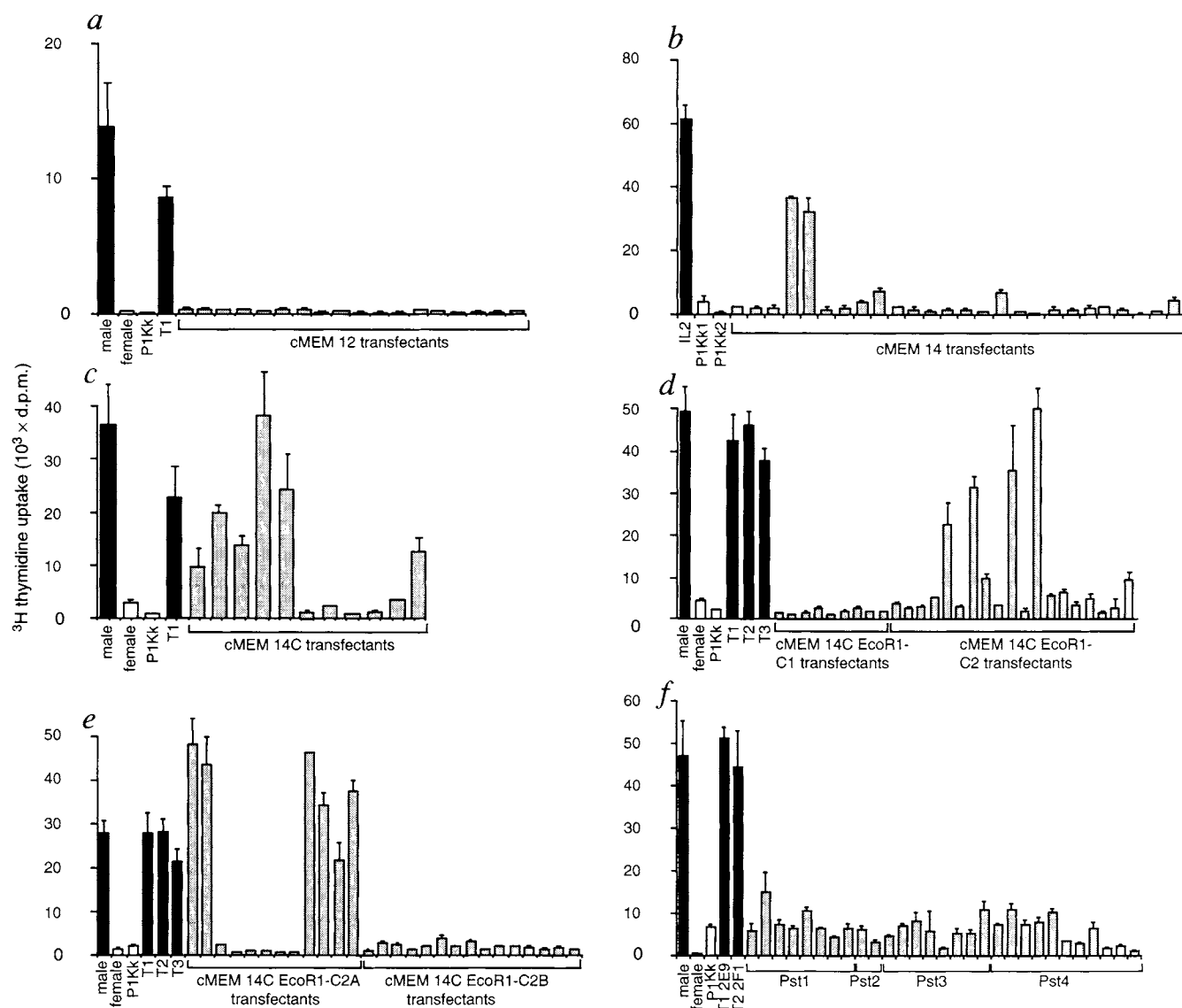


FIG. 2 Identification of a cosmid cMEM 14 and its subcloned fragments that express H-YK^k. a, P1K^k cells transfected with cosmid cMEM 12 fail to stimulate C6, the H-YK^k T-cell clone. Responses to positive controls (male and positive transfectant cells; T1, cMEM 14; T2, cMEM 14C; T3, cMEM 14CRI (unfractionated *Eco*RI-digested cMEM 14C) and interleukin-2) are shown as filled bars; negative controls (female and untransfected P1K^k cells), open bars; individual clones of transfected P1K^k cells, grey bars. b, Cells transfected with cosmid cMEM 14 express H-YK^k and stimulate 2A11, the H-YK^k-specific T-cell hybridoma. c, Cells transfected with the subcloned *Sac*I fragment, cMEM 14C, stimulates C6. d, Localization of H-YK^k to the *Eco*RI-C2 subcloned fragment of cMEM 14C. e, Identification of a 1.1-kb *Eco*RI/*Eco*RV subcloned fragment cMEM 14C *Eco*RI-C2A, which expresses H-YK^k on transfection. f, None of the four subcloned *Pst*I fragments of cMEM 14C express H-YK^k when transfected into P1K^k cells.

METHODS. P1Htr cells (5×10^6) expressing H-2K^k MHC class I molecules²⁷ were cotransfected with the cosmid or subcloned fragment (10 μ g) and 1 μ g pH β APr-1-neo²⁸ by electroporation or CaPO₄²⁷. Stably transfected antibiotic-resistant cells were assayed using the H-YK^k-specific T-cell clone C6¹⁸ or hybridoma 2A11 (ref. 12). For C6, 5×10^4 transfectant cells were incubated with 10^4 T cells in 200 μ l RPMI 1640 medium containing hypoxanthine, aminopterin (HA) and 5 ng ml⁻¹ PMA in 96-well FB plates for 72 h. The cells were pulsed with ³H thymidine for the last 6–18 h of incubation, collected using a Tomtec 96-well plate harvester and counted using a Wallac 1205 Betaplate counter. Results are expressed as disintegrations per minute ($10^3 \times$ d.p.m.). For 2A11, 2.5×10^4 transfectants were mixed with 10^4 hybridoma cells in 200 μ l RPMI-1640 in 96-well FB plates for 24 h. Supernatant (100 μ l) was removed and added to an interleukin-2 responsive T-cell clone. After incubation for 48 h the T cells were pulsed, collected and counted as described above.

ized the H-YK^k epitope to a 1.1-kilobase (kb) *EcoRI*–*EcoRV* DNA fragment. This fragment, designated C2A, includes the 3' end of exon 2, the whole of exon 3 and most of exon 4 (Fig. 1c), the large exon. *PstI* digestion of cMEM 14C completely disrupted H-YK^k epitope expression (Fig. 2f).

To identify the H-YK^k epitope precisely, we compared the amino acid sequences encoded by the C2A fragment of *Smcy* with the same region of *Smcx* (Fig. 3a). Six peptides conforming to the H-2K^k binding motif XEXXXXXX²⁰, or similar sequences XEXXXXX(X)L/V, were screened for their ability to confer H-YK^k expression when presented by PIK^k cells. Peptide 6 (amino acids 244–251) expresses the H-YK^k epitope, with maximal stimulation at concentrations of 100 pM to 10 nM (Fig. 4a, c), which is similar to other peptide antigens. Peptide 3 (amino acids 104–111) also stimulated, but only at a concentration of 10 µM. Although peptide 3 required at least a 1,000-fold higher concentration to stimulate the T-cell clone than peptide 6, it was derived from a DNA sequence that spans one of the *PstI* sites, and *PstI* digestion led to loss of H-YK^k expression (Fig. 2f). We therefore used 3' deletion mutants of the C2A fragment to distinguish which peptide defined the H-YK^k epitope. Only cells transfected with the undeleted construct ('del 3') expressed H-YK^k (Fig. 4b). Cells transfected with the two constructs ('del 1' and 'del 2') that were deleted from positions 193 or 227, but which still expressed peptide 3 (Fig. 3a), failed to stimulate the H-YK^k-specific T-cell clone. (Expression was confirmed by RT-PCR; data not shown.) Thus the H-YK^k epitope expressed by *Smcy* is the peptide TENSCKDI.

To determine which residues were important in defining the T-cell epitope, a series of synthetic peptides substituting the *Smcx*-encoded amino acid at various positions were tested for H-Y expression. The *Smcx* peptide itself did not stimulate the T-cell clone, neither did an octamer with K added at position 6 (Fig.

4c). Introducing the *Smcx* amino acid at positions 1 or 3 caused some loss of potency, whereas substitution at positions 4 or 7 caused a 1,000-fold shift in the concentration required for maximal stimulation. These substitutions did not affect the two anchor residues (E-2 and I-8) that are important in binding to the MHC class I molecule, suggesting that residues 4 and 7 may be important in T-cell recognition.

Our results identify a Y-chromosome gene that expresses an H-Y epitope and defines a genetic basis for the antigenic difference between males and females. Previously, an H-YD^b epitope has been eluted as a mixture of peptides from male cells⁶. In addition, several candidate peptides that could mimic the natural H-YD^b peptide have since been identified from a random peptide library²¹, but in neither case was the natural peptide defined or the gene encoding this epitope identified. *Smcy* contains one H-Y epitope. Whether the other H-Y epitopes are expressed by *Smcy* or by other genes in this region is uncertain. cMEM 14 transfectants expressing H-YK^k did not express H-YA^b, but as it is not understood how these epitopes are expressed these negative findings need to be interpreted with caution. To elicit a graft rejection response, minor transplantation antigens need to express at least two separate epitopes which can be encoded by separate genes^{22,23}. We have previously shown that, for H-Y, different epitopes may also be expressed from separate genes^{12,19}. Thus H-Y antigens may act as markers for as yet unrecognized Y-chromosome genes.

The expression of the H-YK^k epitope from an exon at the 3' end of the *Smcy* gene in the absence of an endogenous promoter is similar to findings reported for tumour transplantation antigens^{16,17}. The mechanisms controlling expression of these subgenomic fragments are not understood, but both intronic sequences preceding the exon expressing the epitope and in-frame ATGs in the exon itself are required²⁴. The inability of our transfectants to express H-YK^k after *PstI* digestion could

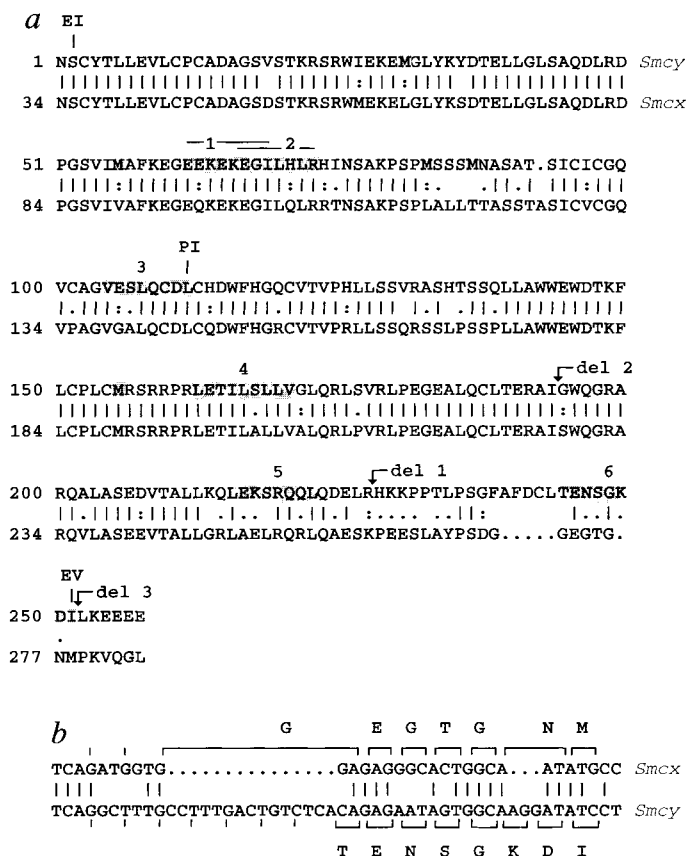


FIG. 3 Identification of potential H-YK^k peptides in the cMEM 14C *EcoRI*-C2A fragment. **a**, Comparison of the predicted amino acid sequences of cMEM 14C *EcoRI*-C2A exons and the homologous sequence from mouse X, *Smcx* cDNA. Six candidate peptides expressing the H-2K^k binding motif (XEXXXXXX)²⁰ or similar sequences (XEXXXXX(X)L/V) and differing from *Smcx* by at least one amino acid are highlighted. The five methionine residues representing possible translational initiation codons are also highlighted. The positions of the *EcoRI*, *PstI* and *EcoRV* sites from the nucleotide sequence are shown as EI, PI and EV, respectively. Arrows indicate the positions at which the three deletion mutants, del 1, 2 and 3, terminate. In the sequence comparison, the vertical lines indicate sequence identity; :, conservative substitutions, and ., non-conservative substitutions. **b**, Comparison of the cDNA and predicted amino acid sequences of *Smcy* and *Smcx* in the region where the H-YK^k peptide is located.

METHODS. The *Smcx* and *Smcy* cDNAs were isolated from a 129/Pas adult testis cDNA library⁸. Sequencing was performed as described¹⁴ using exonuclease III/mung bean nuclease generated nested deletions.

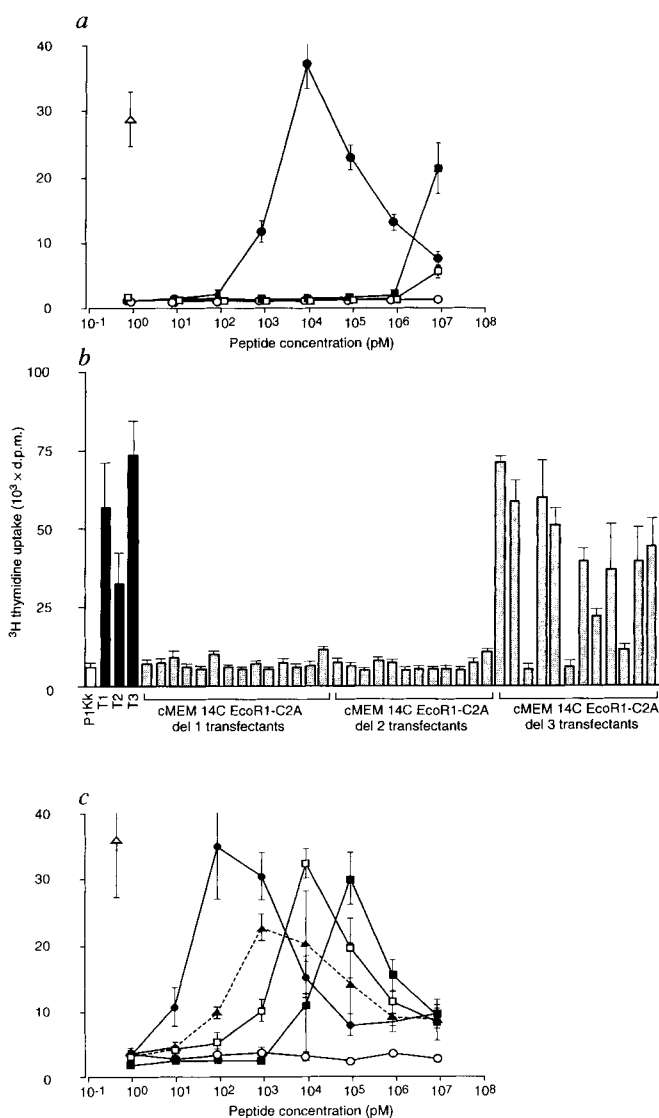


FIG. 4 Identification of the peptide expressing the H-YK^k epitope and effects of sequence modification on its ability to stimulate the H-YK^k-specific T-cell clone, C6. **a**, The six peptides identified in Fig. 3a were tested for their ability to stimulate the T-cell clone, C6. The open triangle shows the control stimulation by CBA male cells; open circles, peptides 1, 2 and 5 at the concentrations given on the x-axis; filled squares, peptide 3; open squares, peptide 4; and filled circles, peptide 6. **b**, Deletion mutants expressing peptide 3 (amino acids 104–111) but not peptide 6 (244–251) no longer confer H-YK^k epitope expression. Transfectants were screened using the T-cell clone, C6. Experimental details are as described in Fig. 2. **c**, The effects of substituting *Smcx* amino acids at various positions of TENSCKDI on its ability to stimulate the T-cell clone, C6. The open triangle shows the stimulation by control CBA male cells; open circles, the 7-mer X peptide and an 8-mer X peptide with K added at position 6, at the concentrations shown on the x-axis; filled circles, the Y peptide; open squares, the G-1 substituted peptide; filled triangles, peptide G-3; filled squares, peptides, T-4 and G-7. **METHODS.** Peptides were synthesized using Fmoc-protected amino acids and (2-(1-H-Benzotriazol-2-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) activation chemistry. After purification by HPLC the fidelity of synthesis was confirmed by mass spectrometry. Synthetic peptides were made up as 1 mM sterile stocks, in PBS for peptides 1, 2, 3, 5 and 6, or methanol for peptide 4. Serial tenfold dilutions were made in triplicate. P1K^k cells (2.5×10^4) were added with 10^4 C6 T-cell clone in RPMI 1640 medium containing HA and 5 ng ml⁻¹ PMA. The cells were incubated for 72 h and pulsed and collected as described in Fig. 2. 3' deletions were made in the cMEM 14C EcoRI-C2A fragment using a double-stranded nested deletion kit (Pharmacia) after digestion with *EcoRV* and *KpnI*. The deletions were characterized by *EcoRI* digestion and by PCR using primers specific for the T₃ and T₇ promoters of the pKS vector. The three selected deletion mutants were sequenced directly from the double-stranded plasmid using the dideoxy chain termination method.

be due to the loss of intronic sequences preceding exon 4. Alternatively, *PstI* digestion might cause the loss of four of the five in-frame ATGs in this region (Fig. 3a), one of which could act as the translational initiation codon within this fragment.

Smcy and *Smcx* share strong amino-acid sequence homology with the retinoblastoma binding protein RBP2 (refs 10, 11, 25). There is more than 65% sequence identity in the amino-terminal half of these proteins where zinc-finger and homeobox motifs are found. Thus, like RBP2, *Smcy* may be involved in the regulation of gene transcription. In the carboxy-terminal region there is much less homology between the sequences, and the RB protein binding motif is absent in *Smcy* and *Smcx*. The H-YK^k epitope

is located in this region. Here *Smcy* and *Smcx* encode dissimilar peptides (Fig. 3b). The sequence of the *Smcy* peptide, however, is similar to that of the human X homologue, XE169 (ref. 26), suggesting that this represents the ancestral form of the protein. Surprisingly, it is *Smcx* and not *Smcy* that has diverged here. Thus, in the mouse, this region has been conserved only on the Y chromosome, suggesting that the H-YK^k domain of *Smcy* has a male-specific function, perhaps as a component of *Spy*. The identification of this H-Y antigen will help elucidate its function, and has implications for therapeutic approaches to transplantation rejection responses directed against minor histocompatibility antigens. □

Received 20 June; accepted 26 July 1995.

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ACKNOWLEDGEMENTS. We thank D. Woods, J. Opp and G. Longpied for technical assistance; P. J. Dyson for advice; P. Byfield for providing the synthetic peptides; A. Mellor, S. Rastan and J. Scott for comments on the manuscript; V. Tikerpa for secretarial assistance, and J. Fallows for preparation of the figures.

Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos

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THE *Drosophila* homologue of the mammalian epidermal growth factor (EGF) receptor (DER)^{1,2} is a receptor tyrosine kinase involved in many stages of fly development, including photoreceptor determination, and wing-vein formation³⁻⁹. Its primary activating ligand is the Spitz protein^{10,11}, which is similar to mammalian TGF- α (ref. 12). Argos is a secreted protein that, like Spitz, contains a single EGF motif¹³⁻¹⁵. It is a repressor of cell determination in the eye, and acts in other tissues, including the wing¹⁶⁻¹⁸. Because Argos has the opposite effects to DER in the eye (the former blocks photoreceptor determination, the latter promotes it) we have tested whether it acts by blocking the DER pathway. We show that Argos does indeed repress this pathway *in vivo* and find that, *in vitro*, Argos protein can inhibit the activation of DER by Spitz. Thus the determination of cells by the DER pathway is regulated by a balance between extracellular activating and inhibiting signals. This is the first *in vivo* example of an extracellular inhibitor of a receptor tyrosine kinase.

To test the hypothesis that Argos acts by inhibiting EGF receptor signalling, we looked for genetic interactions between mutations in *argos* and genes of the EGF receptor signalling pathway^{19,20}. Interactions in the eye were seen between *argos* and every known member of the pathway: when we halved the gene dosage of components in the DER pathway, the *argos* hypomorphic (reduction in function) phenotype was suppressed (Table 1). An example is shown in Fig. 1*a, b*: halving of *spitz* causes the irregular ommatidial array of *argos*^{el133} to revert to near wild-type. Conversely, the same reduction in *spitz* enhances the rough-eye phenotype caused by *argos* overexpression (Fig. 1*c, d*).

To substantiate the genetic interactions seen in the eye, we have studied Argos function in the developing wing, where the EGF receptor also functions⁹. One *argos* hypomorphic allele has been reported to produce extra vein material¹⁶, and on close examination we have found that all *argos* hypomorphic mutations have an extra-vein phenotype, although the severity and penetrance is variable. Strikingly, the exact position and amount of extra vein material seen (Fig. 1*e*) is very similar to that seen in gain-of-function alleles of *DER* (Fig. 1*h*). Furthermore, the loss of vein phenotype seen when *argos* is slightly overexpressed (Fig. 1*f*) occurs in an identical position on vein L4 to that characteristic of *DER* hypomorphs (Fig. 1*i*); compare these phenotypes with a wild-type wing (Fig. 1*g*). These converse wing phenotypes also suggest that Argos acts to inhibit EGF receptor function. As in the eye, we have detected extensive interactions between *argos* and mutations in the DER pathway in the wing (Table 1). The results are similar: the phenotype of *argos* loss is suppressed by halving the dose of members of the DER pathway (Fig. 1*j*); the phenotype of *argos* overexpression is enhanced by the same mutations (results not shown). Furthermore, overexpression of the EGF receptor can overcome the defects caused by *argos* overexpression (Fig. 1*k-m*). All of these observed interactions support the conclusion that Argos inhibits EGF receptor signalling *in vivo*.

TABLE 1 Summary of genetic interactions seen in eyes and wings between *argos* and members of the EGF receptor signalling pathway

Argos reduction phenotype is suppressed by decrease in DER signalling pathway
Argos overexpression phenotype is enhanced by decrease in DER signalling pathway, and suppressed by increased DER signalling
Decrease in DER signalling phenotype is suppressed by decrease in Argos
Hyperactive DER pathway signalling is enhanced by decrease in Argos, and suppressed by Argos overexpression

Genetic interactions of the kind indicated were seen between *argos* and every member of the EGF receptor signalling pathway that was tested, that is, in the order of the pathway, *spitz*, *star*, *rhomboid*, *DER*, *drk*, *Sos*, *Gap1*, *Ras1*, *raf*, and *rolled* (encoding MAP kinase). All these interactions point to the same general conclusion: Argos acts to repress the activity of the EGF receptor pathway. The following alleles of genes in the DER/Ras1 signalling pathway were tested: *spitz*^{A14}, *spitz*^{OE92}, *spitz*^{T25}, *S*²¹⁸, *S*⁵⁶⁷¹, *rho*^{PAS}, *drk*^{EOA}, *Sos*^{e2H}, *Sos*^{EOO}, *Gap1*^{B2}, *Gap1*^{S16}, *Ras1*^{e2F}, *Ras1*^{e1B}, *raf*¹¹²⁹, *raf*^{HM7}, *rj*^{10a}, *rj*²⁶ and *rj*¹. DER mutations tested were: *flb*^{1K35}, *flb*^{1P02}, *flb*^{2W74}, *flb*^{1F26}, *flb*^{2EO7}, *flb*^{2X51}, *flb*^{3B41}, *flb*^{2L65}, *top*¹⁰¹, *Elp*^{B1} and *Elp*^{B1}. The nomenclature of these alleles follows the conventions of earlier publications. *argos*^{IA7} (ref. 13) was used as a null allele. *argos*^{el133} (ref. 14) was used as a hypomorphic eye allele, and *argos*^{VA9} (M.F., unpublished) as a hypomorphic wing allele. HS-*argos* line 4 (ref. 16) was used for Argos overexpression. N.B. The mutation *giant lens* (*gil*) is a synonym of *argos*.

As Argos is a secreted protein containing a single EGF domain¹³, the simplest explanation for the inhibition seen *in vivo* is that it is a direct inhibitor of ligand-induced EGF receptor activation. We have tested this prediction in a cell-culture system. The first step of the signal transduction cascade triggered by EGF receptor activation is the transphosphorylation of tyrosine residues in the intracellular domain of receptor dimers²¹. This activation can be detected in a *Drosophila* S2 cell line that expresses DER: after incubating these cells with secreted Spitz protein, DER is immunoprecipitated from cell extracts, and the amount of tyrosine phosphorylation is assayed on western blots. There is a low level of tyrosine phosphorylation of the EGF receptor in the absence of ligand, but addition of Spitz causes a rapid and substantial increase in EGF receptor phosphorylation¹¹. We found that Argos protein is an effective inhibitor of this activation (Fig. 2*a*): preincubation of the cells with Argos (for only 2 min) prevents any detectable activation by Spitz. Quantification of western blots indicates that activation of the EGF receptor by Spitz, and its inhibition by Argos, are both saturable responses, and they are competitive: a given concentration of Spitz is increasingly blocked by increasing Argos concentration, and the inhibition shown by a particular level of Argos can be overcome by increasing the concentration of Spitz (Fig. 2*b*).

The effect of Argos on EGF receptor activation can also be demonstrated in the absence of Spitz. When DER is overexpressed in cell culture, a significant amount of autophosphorylation is seen²², presumably caused by spontaneous dimerization of the receptor when it is present at high concentrations in the plasma membrane. We found that Argos significantly reduces the level of this ligand-independent activation (Fig. 2*c*), implying that Argos does not block activation through interaction with Spitz but instead acts directly on the DER-expressing cells. Our results are not consistent with Argos acting to reduce EGF receptor expression: first, the inhibition is too rapid; second, the DER gene is expressed under a foreign promoter in the tissue culture assay; and third, the level of DER remains constant during all our assays (bottom panels of Fig. 2*a, c*). We therefore conclude that Argos is acting as an extracellular inhibitor of the activation of DER by Spitz.

Because Spitz is a potent activator of the EGF receptor¹¹, and the activation of its pathway can trigger a wide variety of cell fates, it follows that tight regulation of this system is crucial for

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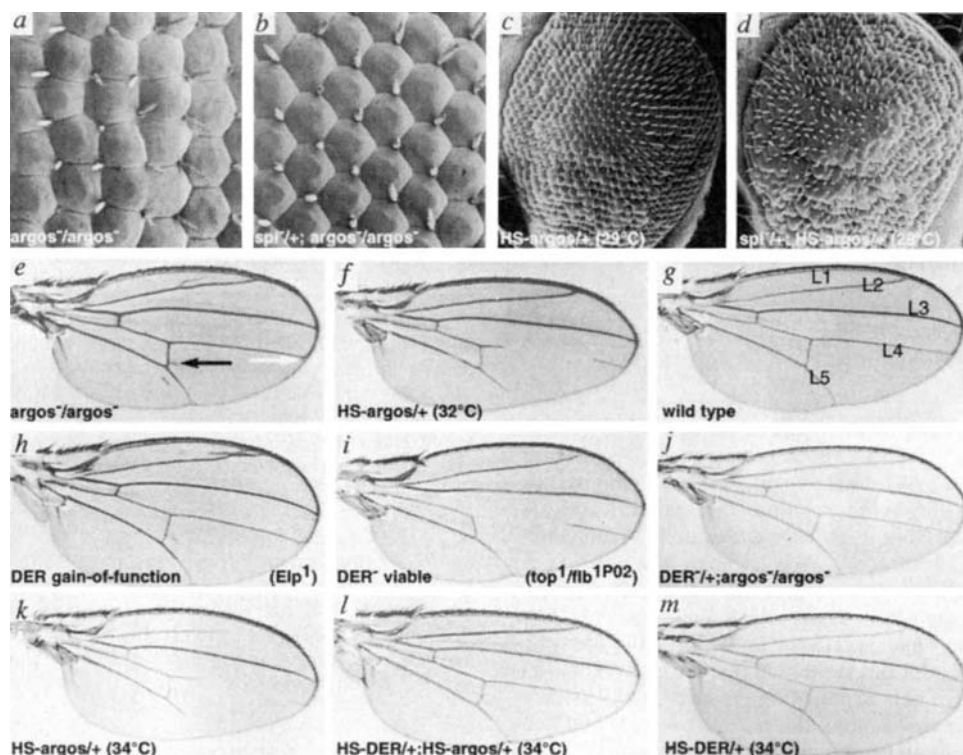


FIG. 1 Genetic interactions of *argos* with EGF-receptor-signalling components. *a–d*, Scanning electron micrographs of fly eyes of the following genotypes. *a*, *argos^{EL133}/argos^{EL133}*. *b*, *spi^{A14}/+; argos^{EL133}/argos^{EL133}*. *c*, *HS-argos/+*. *d*, *spi^{A14}/+; HS-argos/+*. All flies were grown at 29 °C. *a*, *argos^{EL133}* flies have rough eyes at 29 °C; the regular hexagonal array of facets is disrupted. *b*, Removal of one copy of the *spitz* gene (halving the level of Spitz) suppresses the *argos* phenotype, bringing the eye back to close to wild-type. (Remember that *spitz* is completely recessive so that a half dose in a wild-type background has no effect.) *c*, At 29 °C, *HS-argos/+* gives slightly disrupted eyes. This is enhanced by halving the dose of *spitz* (*d*). *e–m*, Wings of the following genotypes are shown. *e*, *argos^{VA9}/argos^{VA9}*. *f*, *HS-argos/+*, heatshocked at 32 °C. *g*, Wild type. *h*, *Elp¹/+*. *i*, *top¹/flb^{1P02}*. *j*, *flb^{1K35}/+; argos^{VA9}/argos^{VA9}*. *k*, *HS-argos/+*, heatshocked at 34 °C. *l*, *HS-DER/+; HS-argos/+*, heatshocked at 34 °C. *m*, *HS-DER/+*, heatshocked at 34 °C. *e*, *argos* loss-of-function mutants cause extra wing vein material. Typically this occurs

at the distal portion of L2, and often some is found adjacent to the posterior crossvein (black arrow); frequent broadening of veins where they join the margin is also seen (white arrow); compare with wild-type in *g*. This phenotype is similar to that caused by the EGF receptor gain-of-function allele, *Elp¹*, shown in *h*. *f*, Overexpression of Argos causes the opposite phenotype: veins are missing. When overexpressed slightly, a characteristic region of L4 is lost; this corresponds to the region lost when the function of *DER* is reduced (*i*). *j*, Halving the *DER* dose (which has no effect in wild-type flies) completely suppresses the *argos^{VA9}* wing phenotype (compare with *e*). *k–m*, The phenotype of overexpressing Argos (*k*) is suppressed by also overexpressing the EGF receptor (*l*); under these conditions the *HS-DER* flies have no phenotype (*m*). See Table 1 for a summary of interactions and a list of alleles used in this study. In all cases where an *HS-argos* or *HS-DER* construct was used, the heat shock regime was 1 h every 6 h at the indicated temperature.

normal development. Negative regulation by Argos provides a mechanism for accomplishing this control. Given the common EGF motif in these proteins, Argos may act as a direct inhibitor of binding of Spitz to the EGF receptor, by competing for the same binding site (Fig. 3*a*). Interestingly, one of the cysteine loops in the Argos EGF repeat is significantly longer than that found in other examples of this motif¹³. Consistent with the view proposed in Fig. 3*a* is the requirement of an intact EGF domain in Argos for its function: a mutant form in which cysteines 4 and 5 of the repeat have been replaced by serine, and a highly conserved glycine (Gly 410) by alanine, is non-functional *in vivo*, as assayed by its ability to rescue the *argos* eye phenotype. However, direct binding of Argos to the EGF receptor, and competition with Spitz, have yet to be demonstrated, and other mechanisms are possible. Note that the presence of an EGF repeat cannot be taken as a prediction of binding to the EGF receptor, as these common motifs mediate a variety of protein–protein interactions. Argos could inhibit EGF receptor function by binding to an unknown receptor (expressed appropriately during development and in S2 cells), which then blocks *DER* activation, for instance by stimulating a tyrosine phosphatase activity, or by producing non-signalling heterodimers (Fig. 3*b*). It has been reported that platelet-derived growth factor can repress EGF receptor signalling in some mammalian cell lines,

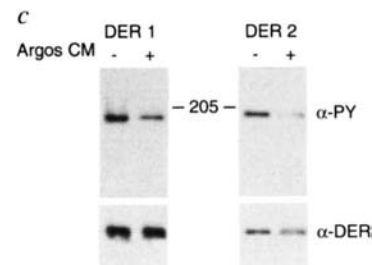
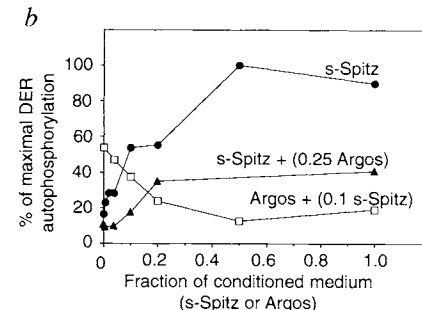
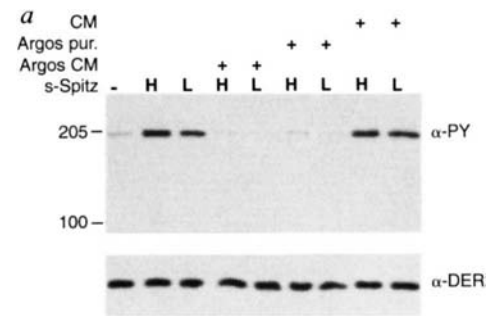
by a mechanism dependent on protein kinase C (PKC)^{23,24}. Argos inhibition of *DER* is not similar to this ‘transmodulation’ effect because we find no involvement of PKC in the process (see legend to Fig. 2).

Several roles for *in vivo* regulation of EGF receptor signalling by Argos can be envisaged. Argos could act simultaneously with the activating ligand(s), so that the balance between the concentration of Argos and these ligand(s) would determine the level of *DER* signalling. Alternatively, activating ligand(s) and Argos could work sequentially, so that activation of *DER* would induce the expression of Argos, which would in turn be responsible for shutting off signalling: a negative feedback loop. We favour this latter view because we find that in the developing embryo and in cell culture Argos expression is dependent on EGF receptor signalling (M. Golenbo, R.S., M.F. and B.-Z.S., submitted). Consistent with this, Argos can inhibit previously activated EGF receptor in tissue culture (Fig. 2*c* and results not shown).

Inhibitory ligands of the interleukin-1 and melanocyte-stimulating hormone receptors have been described^{25,28}. Argos is, however, the first reported example of an extracellular inhibitor of a receptor tyrosine kinase that acts *in vivo* as well as *in vitro*. Structure–function analysis of mammalian EGF and TGF- α has failed to uncouple the binding and activation functions of these ligands. Therefore attempts to produce antagonistic analogues

FIG. 2 Argos inhibits activation of the EGF receptor by Spitz. **a**, DER activation was assayed by measuring the levels of tyrosine phosphorylation of the M_r 180K EGF receptor immunoprecipitated from cell extracts. S2:DER2f cells²¹ show a low level of spontaneous autophosphorylation (left-hand lane). Following addition of secreted Spitz (sSpitz) protein at high (H) or limiting (L) concentrations, high or intermediate levels of DER activation were observed, respectively. Preincubation of the S2:DER2f cells with Argos protein in conditioned medium from baculovirus-infected Sf9 cells (Argos CM) or with the purified Argos protein (Argos pur.) resulted in complete inhibition of EGF receptor activation by Spitz. Preincubation with a control Sf9-conditioned medium (CM) did not affect the ability of the secreted Spitz protein to activate the EGF receptor. Argos alone causes no EGF receptor activation, and no observable increase in tyrosine phosphorylation on any other protein (not shown). The lower panel shows a western blot of an identical gel probed with anti-DER antibodies: the DER levels in each sample were similar. We also found that an unrelated six-histidine-tagged protein was unable to block activation (results not shown). In some mammalian cell lines, platelet-derived growth factor (PDGF) represses the activation of the EGF receptor, so-called 'transmodulation'²³. This effect is variable and not fully understood, and its biological significance is unclear, because PDGF enhances EGF action on cell growth. Transmodulation occurs through protein kinase C (PKC), and can be mimicked by phorbol esters, PKC activators²⁴. Stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) has no effect on the Spitz activation of DER. Extended incubation (6 or 16 h) with PMA depletes PKC activity, and this treatment did not affect Argos inhibition; neither did incubation of the cells with GF 109203X, a specific PKC inhibitor. We therefore conclude that Argos acts by a mechanism distinct from transmodulation. **b**, Dose-response curves for the competition between Argos and secreted Spitz. Filled circles, incubation of S2:DER2f cells in increasing concentrations of the Spitz medium (diluted up to 1/200); filled triangles, preincubation in Argos medium diluted 1/4 for 2 min followed by increasing concentrations of Spitz; open squares, preincubation in increasing concentrations of Argos medium followed by addition of Spitz diluted 1/10 for 5 min. The activation of the EGF receptor by Spitz is saturable, as is the inhibition by Argos. It has not so far been possible to purify active Spitz protein from the conditioned medium, which sets a rather low upper limit on the Spitz concentration available for these competition curves. **c**, There are two N-terminal variants of the *Drosophila* EGF receptor²: overexpression of either form has previously been shown to result in ligand-independent activation²². Incubation with Argos decreased the level of EGF receptor tyrosine phosphorylation seen in both cases after 10 min, indicating that Argos also inhibits ligand-independent autoactivation of the receptor.

METHODS. The EGF receptor activation assay was performed as previously described¹¹. Dilutions of 1/10 or 1/50 were used for the high or limiting Spitz levels, respectively. The DER protein was immunoprecipitated and parallel western blots were probed by anti-phosphotyrosine antibodies to check the level of DER activation, and with anti-DER antibodies to verify that equal amounts of the DER protein were used for all the treatments. Recombinant Argos protein, with a six-histidine tag introduced between Pro 29 and Leu 30, was made in the baculovirus expression system. The tagged gene was cloned into pVL1393 (Pharmingen), and the protein produced in Sf9 cells grown in CCM3 serum-free medium (Hyclone), where it accumulated to approximately $5 \mu\text{g ml}^{-1}$. The protein was purified by Ni-NTA affinity chromatography



(Qiagen). S2:DER2f cells were preincubated for 2 min in the His6-Argos conditioned medium or the purified protein added at the same Argos concentration to Schneider's medium. Subsequently, the secreted Spitz protein was added for 5 min. The control conditioned medium came from Sf9 cells infected with an irrelevant baculovirus. For the dose-response competition curves, western blots were detected with ¹²⁵I-labelled protein A (Amersham), and the signal intensity was quantified with a phosphorimager (Fuji). The intensity of each band was calculated by subtracting the general gel background reading. On each gel a lane of DER activated by secreted Spitz diluted 1/10 was used as an internal standard, and each blot was normalized to this baseline. The concentrations of Spitz and Argos proteins in the conditioned media we have used are comparable (10–100 nM).

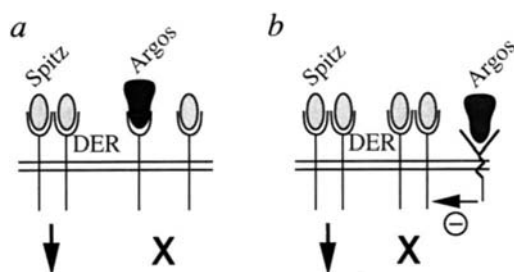


FIG. 3 Models of Argos function. **a**, The simplest interpretation of our data is that Argos competes with Spitz for binding to the EGF receptor, thereby blocking its ability to dimerize and/or signal efficiently. **b**, An alternative view is that Argos binds to an unknown receptor, thus indirectly inhibiting EGF receptor signalling. Further biochemical characterization should differentiate between these two models.

have been largely unsuccessful²⁹. The conservation of the EGF-receptor signalling pathway between flies and mammals³⁰ raises the possibility that a mammalian homologue of Argos exists, and it will be interesting to see whether similar extracellular inhibition of the EGF receptor occurs in vertebrates. Furthermore, the possibility of engineering Argos-like inhibitors in mammals could be of theoretical and clinical interest. □

Received 10 May; accepted 13 July 1995.

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ACKNOWLEDGEMENTS. R. Schweitzer and R. Howes contributed equally to this work. We thank M. Golebo and R. Seger at the Weizmann Institute of Science, and many colleagues at the MRC Laboratory for discussions and advice. This work was supported by grants from the NIH, the Council for Tobacco Research, the DKFZ and the Israel Academy of Science (B.S.), and by the Medical Research Council (M.F.). R.H. holds an MRC postgraduate studentship.

Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway

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In the budding yeast *Saccharomyces cerevisiae*, the process of conjugation of haploid cells of genotype *MATa* and *MATa* to form *MATa/a* diploids is triggered by pheromones produced by each mating type. These pheromones stimulate a cellular response by interaction with receptors linked to a heterotrimeric G protein. Although genetic analysis indicates that the pheromone signal is transmitted through the G $\beta\gamma$ dimer, the initial target(s) of G protein activation remain to be determined. Temperature-sensitive cells with mutations of the *CDC24* and *CDC42* genes, which are incapable of budding and of generating cell polarity at the restrictive temperature^{1–3}, are also unable to mate⁴. Cdc24 acts as a guanylyl-nucleotide-exchange factor for the Rho-type GTPase Cdc42⁵, which has been shown to be a fundamental component of the molecular machinery controlling morphogenesis in eukaryotic cells^{6–10}. Therefore, the inability of *cdc24* and *cdc42* mutants to mate has been presumed to be due to a requirement for generation of cell polarity and related morphogenetic events during conjugation. But here we show that Cdc42 has a direct signalling role in the mating-pheromone response between the G protein and the downstream protein kinase cascade.

In order to determine whether the role of Cdc24/Cdc42 in mating is limited to morphogenetic functions, we determined the integrity of other aspects of the mating-pheromone response in *cdc24* and *cdc42* temperature-sensitive (*ts*) mutants. Induction of pheromone-responsive genes such as *FUS1* constitutes one of the immediate responses to the pheromone signal and can occur at all stages of the cell cycle except late G1¹⁷. Figure 1a shows

that *cdc24* and *cdc42* mutants shifted to their restrictive temperature were unable to induce a *FUS1-LacZ* fusion gene after addition of α -factor. Thus, inactivation of Cdc24 or Cdc42, in addition to interfering with morphogenetic events required for mating, prevents gene induction by pheromone, suggesting a role for these proteins in the signal transduction pathway itself. If Cdc24 and Cdc42 are required for mating-pheromone signalling, then inactivation of the proteins encoded by *ts* alleles should also release pheromone-arrested cells from their G1 block. To test this, *cdc24*, *cdc42* and wild-type cells were arrested in G1 at permissive temperature by treatment with α -factor, after which they were shifted to restrictive temperature while continuing incubation with the mating pheromone. Flow cytometric analysis showed that wild-type cells remained arrested in G1, as expected, but that *cdc24* and *cdc42* cells underwent DNA replication, indicating release from the G1 block (Fig. 1b). This result indicates that continued activity of Cdc24 and Cdc42 is required to maintain the pheromone signal after it has been established.

If activation of Cdc42 by guanylyl nucleotide exchange is required for pheromone signalling, mutational activation of Cdc42 might be expected to enhance the pheromone response. Expression of the activated *CDC42*^{Val12} allele before addition of α -factor led to hyperinduction of a *FUS1-lacZ* reporter, reflecting a potentiation of the pheromone response pathway compared to the parental strain (Fig. 2a). However, expression of the activated Cdc42 was not sufficient to activate the pathway before the addition of α -factor (Fig. 2a, time 0). A possible explanation for this failure is that Cdc42 function requires mobilization of upstream elements for purposes other than guanylyl nucleotide exchange, for example, for colocalization of the active GTPase with its target. To test this idea, we coexpressed a dominant negative allele of *STE4* (encoding the β -subunit of the heterotrimeric G protein (G β)) simultaneously with *CDC42*^{Val12}, reasoning that this signalling-defective G β might be capable of supplying other essential upstream functions. Coexpression of *STE4dn-1* and *CDC42*^{Val12} triggered a mating-pheromone response detectable using the *FUS1-lacZ* reporter construct in the absence of mating pheromone (Fig. 2b). Thus, activated Cdc42 can initiate a mating-pheromone response *de novo* if other upstream requirements are met.

FIG. 1 Cdc24 and Cdc42 are required for pheromone signalling. a, α -factor-induced *FUS1* expression in *cdc24-1* and *cdc42-1* temperature-sensitive mutant strains. Both *ts* strains and the parental strain, as well as two other *ts* *cdc* mutant strains as controls (*cdc28-13* and *cdc34-3*), were transformed with a centromeric vector containing a *FUS1-lacZ* reporter gene. Wild-type cells (open bars), *cdc24-1* cells (shaded bars, top), *cdc42-1* cells (black bars, top), *cdc28-13* cells (shaded bars, bottom), and *cdc34-3* cells (black bars, bottom) were grown to mid-log phase at 25 °C and shifted to restrictive temperature (36 °C) for 15 min before addition of 60 ng ml⁻¹ α -factor. Aliquots were taken at the indicated times and β -galactosidase activity was measured after permeabilization of cells. Time 0 corresponds to the time of addition of α -factor. Values are means of duplicates for each point. The different maximal levels of *FUS1-lacZ* induction in the two sets of experiments correspond to different reporter plasmids used, which differ by about a factor of two in efficiency. The wild-type control serves as an internal standard. b, Release from pheromone-induced G1 arrest in *cdc24-1* and *cdc42-1* strains. Cellular DNA content was analysed by flow cytometry²⁵ for wild-type (column 1), *cdc24-1* (column 2) and *cdc42-1* (column 3) cell populations growing asynchronously, treated with α -factor (60 ng ml⁻¹) for 3 h at 26 °C, and shifted to 36 °C for 1 and 2 h. The first peak (at 200 arbitrary units) corresponds to cells in G1 (1N DNA content), whereas the second peak (at 400 arbitrary units) corresponds to cells with a 2N (replicated) DNA content. The 1N peak for the wild-type culture arrested with α -factor tended to drift to higher values with time because of increasing cell size and thus autofluorescence; some non-growing cells remained at about 200 arbitrary units forming a small extra peak. METHODS. *cdc24-1*, *cdc42-1*, *cdc28-13* and *cdc34-3* were congenic with our wild-type strain²⁶. Transformations of yeast were according to ref. 29. β -Galactosidase activity was measured according to ref. 30. Miller units are defined as (A₄₂₀ × 1,000)/(A₆₀₀ × V(ml) × t(min)).

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TABLE 1 Two-hybrid interaction between Cdc42 and Ste20

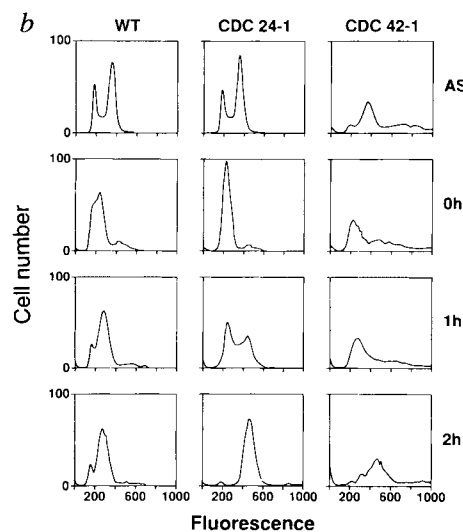
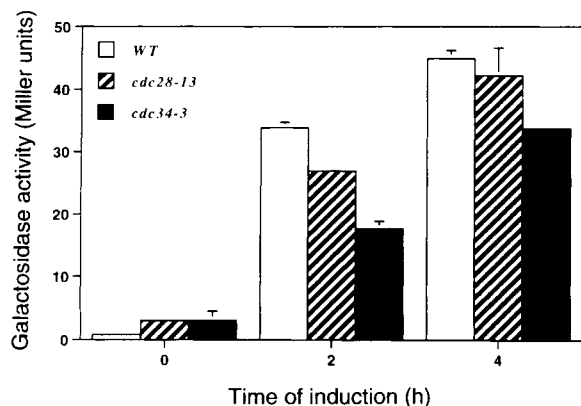
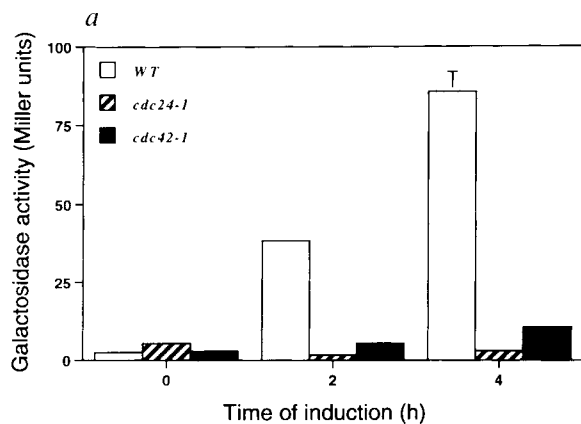
(a) Cdc42 interacts specifically with an amino-terminal domain of Ste20

	pJG4-5-STE20-1	pJG4-5-STE20-2	pJG4-5-STE20-3	pJG4-5-MSB2	pJG4-5
pEG202-CDC42	105	88	232	4	2
pEG202-CDC42 ^{Ser188}	2,111	2,741	3,506	41	40
pEG202-RHO1	10	9	16	7	7
pEG202-RHO1 ^{Ser206}			22	33	30
pEG202-RHO2 ^{Ser188, 189}			28	23	26
pEG202-RHO3 ^{Ser228}			4	14	10
pEG202-RHO4 ^{Ser288}			43	97	69
pEG202-RSR1	21	17	12	10	5
pEG202-RAS2	9	10	7	6	4

(b) Interaction between Ste20 and activated Cdc42

	pJG4-5-CDC42	pJG4-5-CDC42 ^{Ser188}	pJG4-5-CDC42 ^{Val12, Ser188}	pJG4-5-CDC42 ^{Leu61, Ser188}	pJG4-5-MSB2	pJG4-5
pEG202-STE20-4	781 ± 278	2490 ± 500	4895 ± 537	5196 ± 622	20 ± 10	38 ± 3
pEG202-MSB2	2	7	3	3	2	2
pEG202	20	18	26	18	17	17

a, Interaction between Cdc42 and fragments of Ste20 was detected using the two-hybrid system¹⁸. Numbers correspond to average β -galactosidase activities from 3 to 6 different isolates. *CDC42^{Ser188}*, *RHO1^{Ser206}*, *RHO2^{Ser188, 189}*, *RHO3^{Ser228}* and *RHO4^{Ser288}* encode mutant proteins that cannot be prenylated at their C termini and hence are not membrane-associated. METHODS. Interaction between Cdc42 and Ste20 was detected using the two-hybrid system¹⁸ in the configuration described in ref. 19. For construction of the fusions of various GTPase proteins to the LexA DNA-binding domain (DBD) in pEG202, *S. cerevisiae* *CDC42²⁰*, *CDC42^{Ser188}* (ref. 21), *RHO1* and *RHO2* (ref. 22), *RHO3* and *RHO4* (ref. 23), *RSR1* (ref. 5), and *RAS2* (ref. 24) full-length coding sequences were amplified by polymerase chain reaction using Vent DNA polymerase (New England Biolabs). The resulting fragments were subsequently cloned either at the *EcoRI*-*XhoI* site or at the *BamHI*-*XhoI* site of pEG202. The three plasmids pJG4-5-STE20-1, pJG4-5-STE20-2 and pJG4-5-STE20-3, which contain different fragments of STE20 (ref. 12) fused to the activation domain (AD) moiety of pJG4-5, were isolated in a two-hybrid screen for proteins that interact with Cdc42. As a negative control, MSB2 (ref. 27) was amplified by polymerase chain reaction and cloned into pJG4-5. β -Galactosidase activities were measured in three to six different isolates of each strain after growth for 16 h at 30 °C in minimal medium containing 2% galactose and 1% raffinose. The average β -galactosidase activities (in Miller units) are given. b, The two-hybrid system was used as in a except that the protein domains tested for interaction were switched with respect to fusion with the DNA-binding domain and the activation domain. The segment fused to the DNA-binding domain in pEG202-STE20-4 corresponds to amino acids 319–496 of STE20. For construction of the fusions of the wild-type and mutant alleles of Cdc42 to the AD in pJG4-5, *S. cerevisiae* *CDC42*, *CDC42^{Ser188}*, *CDC42^{Val12, Ser188}* and *CDC42^{Leu61, Ser188}* (refs 20, 21) full-length coding sequences were amplified by polymerase chain reaction as described in a. The double mutant genes *CDC42^{Val12, Ser188}* and *CDC42^{Leu61, Ser188}* were amplified by using plasmids containing either *CDC42^{Val12}* or *CDC42^{Leu61}* as templates and a reverse primer that specifically introduces the C to S Ser188 mutation. All numbers are averages of two independent biological experiments performed in triplicate. Standard error is given for the average values of interaction with STE20-4.



Epistasis experiments have suggested that Ste20 functions at the top of a protein kinase cascade that culminates in the MAP kinase homologues Fus3 and Kss1^{12,13}. It is possible, therefore, that Cdc24 and Cdc42 provide a link between the receptor-coupled G protein and Ste20. Two approaches were used to investigate a possible relationship between Cdc42 and Ste20. First, results obtained using the yeast two-hybrid system indicate that

Cdc42 and Ste20 could interact directly *in vivo* (Table 1). The interaction observed was highly specific in that the same Ste20 fusions did not interact with the other small GTPase proteins tested, including four other *S. cerevisiae* Rho-family G proteins, all of which are highly homologous to Cdc42 (Table 1a). In addition, stronger interactions were detected when two different activated alleles of *CDC42*, *CDC42*^{Val12} and *CDC42*^{Leu61}, were

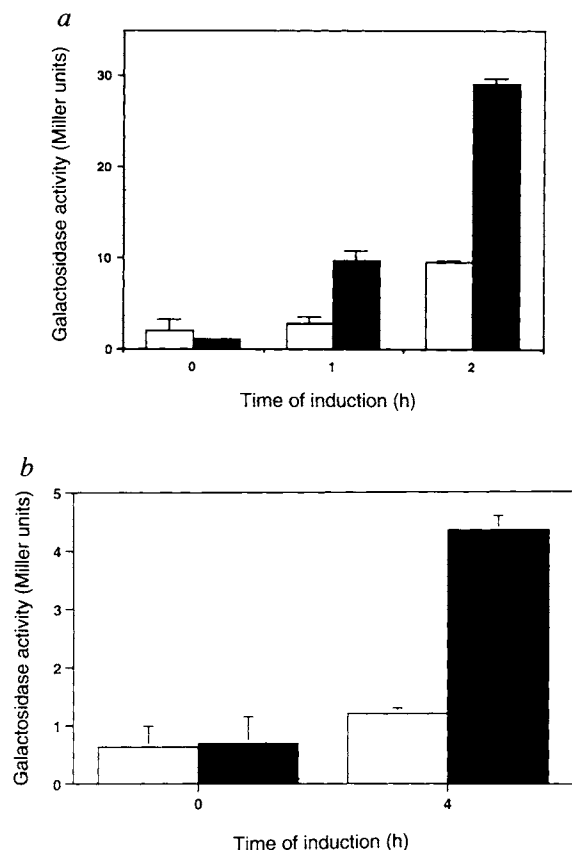
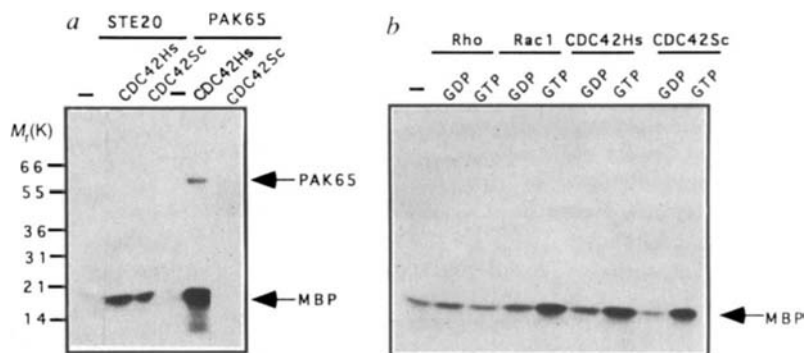


FIG. 2 Effect of *CDC42*^{Val12} expression on the pheromone response. **a**, *CDC42*^{Val12} potentiates the α -factor response. β -Galactosidase activity expressed from a *FUS1-lacZ* reporter gene was measured in the parental strain (open bars) and in the same strain containing a centromeric plasmid with the dominant activated *CDC42*^{Val12} allele under control of the *GAL1* promoter (black bars). Cells were grown in non-inducing sucrose medium to mid-log phase and then transferred to inducing medium containing 2% galactose for 1 h. α -Factor (60 ng ml⁻¹) was added to the culture at time 0 and aliquots were taken at the indicated times for measurement of β -galactosidase activity (see Fig. 1). **b**, *CDC42*^{Val12} can activate the pheromone response *de novo* in the presence of a dominant-negative allele of *STE4*. β -Galactosidase activity from a *FUS1-lacZ* reporter gene was measured in a strain containing a dominant-negative allele of *STE4* (encoding the β -subunit of the receptor-coupled G protein) and *CDC42*^{Val12} both under control of the *GAL1* promoter (black bars). A control strain contained only the dominant-negative *STE4* allele (open bars). Cells were grown in non-inducing sucrose medium to mid-log phase and then transferred to inducing medium containing 2% galactose. β -Galactosidase activity was measured at the time of transfer and after 4 h of induction. Values given represent the averages from two independent experiments; error bars give the variation from the mean. The response, although not equivalent to a maximal pheromone response, indicates complementation of the signalling defect of the mutant *Ste4* by activated *Cdc42*. The dominant negative *STE4* allele (*STE4dn-1*) was isolated from a library of plasmid-borne *STE4* alleles, randomly mutated by PCR, based on its ability to confer dominant α -factor resistance when expressed from the *GAL1* promoter (M.-N.S. and S.I.R., manuscript in preparation). It is presumed that the phenotype conferred by this allele results from an ability to interact with downstream elements in a non-productive fashion.

FIG. 3 GTP-bound Cdc42 activates the Ste20 kinase *in vitro*. **a**, Comparison with PAK65. Purified recombinant yeast Ste20 was incubated either in buffer alone or with purified recombinant yeast or human Cdc42 loaded with GTP- γ S and analysed for myelin basic protein (MBP) kinase activity. In a parallel experiment, human PAK65 (hPAK65), a structural homologue of Ste20, was assayed in the same manner. Although human CDC42 (CDC42Hs) could activate yeast Ste20, yeast Cdc42 (CDC42Sc) could not activate hPAK65. An additional difference between activation of yeast Ste20 and hPAK65 was lack of autophosphorylation observed for Ste20. Because autophosphorylation of hPAK65 (indicated on autoradiograph) renders its activity GTPase independent²⁶, these data suggest that Ste20 may require continued interaction with a GTPase to maintain activity. **b**, Specificity of Ste20 activation. Ste20 was incubated with buffer alone, human RHO1, human RAC1, human CDC42, or yeast Cdc42 either loaded with GTP- γ S or GDP- β S and then tested for the ability to phosphorylate myelin basic protein (MBP; indicated on the autoradiograph). Although a basal level of kinase activity is detected in the absence of added Cdc42, there is a significant GTP-dependent stimulation of this activity by human and yeast Cdc42 and human RAC1. Human RHO1 conferred no stimulation. The GTPase-independent background observed for Ste20 appears to be related to significant contamination with proteolytic fragments containing the catalytic domain but missing the complete amino-terminal regulatory domain and therefore having constitutive activity (data not shown).



METHODS. Recombinant hPAK65 or Ste20 (1–2 μ g bound to protein G-Sepharose conjugated with either myc or glu-glu monoclonal antibodies) was washed with 1 ml and incubated with 40 μ l of kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂) with 1–2 μ g of the indicated GTPase preloaded with either 100 μ M GTP- γ S or GDP- β S. All recombinant proteins used were generated in Sf9 cells as previously described²⁶. The reaction was initiated by adding 10 μ l kinase buffer containing 50 μ M ATP and 5 μ Ci [γ -³²P]ATP and incubated for 20 min at 30 °C. Myelin basic protein (4 μ g) was included in each assay. The reaction was stopped by adding SDS-PAGE sample buffer and boiling for 5 min. Samples were applied to a 14% SDS-PAGE gel which was stained with Coomassie blue, destained, dried and exposed to film.

assayed (Table 1b). The results of the two-hybrid studies are consistent with the demonstration that the highly conserved human homologue of Cdc42 (Cdc42Hs), when in the GTP-bound form, can bind and activate *in vitro* a protein kinase (PAK65) with sequence similarity to Ste20^{14,26}. Second, to characterize further the nature of the interaction between Cdc42 and Ste20, the respective recombinant proteins were expressed and purified. GTP-bound (but not GDP-bound) Cdc42 was found to be capable of stimulating the protein kinase activity of Ste20 *in vitro*, consistent with a direct signalling relationship (Fig. 3).

The genetic and physiological properties of Cdc24 and Cdc42 demonstrated here suggest that these proteins are components of the primary mating-pheromone signal-transduction pathway. These results potentially fill a gap in this signalling pathway, between the heterotrimeric G protein and the downstream MAP kinase cascade, that has remained refractory to elucidation by genetic analysis. The apparent dual role of Cdc42, a Rho-family GTPase, in generation of cell polarity during vegetative growth and in pheromone signal transduction raises the possibility of coregulation of the two processes. We suggest an evolutionarily conserved regulatory motif whereby Rho-family GTPases link cell differentiation to morphogenetic processes. In the yeast mating-pheromone response, polarized growth toward the source of pheromone¹⁵ is correlated with clustering of both pheromone receptors and Cdc42 at the tips of growth projections^{16,17}, presumably oriented by exposure to the pheromone gradient. Thus, Cdc42 would be poised to transduce signals involved in both polarized growth and gene expression. □

Received 2 March, accepted 20 June 1995.

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ACKNOWLEDGEMENTS. We thank G. Mondesert and A. Picard for discussion and for assistance, D. Lew for strains and plasmids, and P. Watts for the library in pJG4-5. M.N.S. was supported by an EMBO long-term fellowship. C.D.V. was supported by the L. and Th. La Roche-Stiftung and Ciba-Geigy-Jubiläums-Stiftung. This work was supported by NIH grants to S.I.R. and J.R.P.

Reconstitution of the initial steps of mitochondrial protein import

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WE have reconstituted the initial steps of mitochondrial protein import with a purified precursor protein, a purified, ATP-dependent, cytosolic chaperone selective for mitochondrial precursors (mitochondrial import stimulating factor; MSF), and either intact mitochondria or intact or solubilized mitochondrial outer membranes. We show that the precursor-MSF complex first binds to the Mas37p/Mas70p subunits of the mitochondrial import receptor. After ATP-dependent release of MSF, the precursor is transferred from Mas37p/Mas70p to the Mas20p/Mas22p subunits of the receptor, and finally delivered to the import channel in the outer membrane. Import in the absence of the MSF bypasses Mas37p/Mas70p. The ATP-mediated transfer of a precursor from MSF to specific subunits of the import receptor is similar to the GTP-mediated transfer of precursors from the signal recognition particle to its receptor on the endoplasmic reticulum.

The import of many proteins into mitochondria is initiated by the interaction of the amino-terminal targeting sequence with MSF, which selectively binds mitochondrial precursor proteins; this interaction causes MSF to hydrolyse ATP¹. The MSF-precursor complex can bind to outer membrane vesicles from rat-

liver mitochondria with concomitant inhibition of ATPase activity².

Here we identify the mitochondrial proteins that recognize the MSF-bound precursor. Because the protein import receptor of mammalian mitochondria is unknown, we have studied the interaction of a precursor-MSF complex with yeast mitochondria. In the yeast *Saccharomyces cerevisiae* the mitochondrial protein import receptor is composed of at least four outer membrane proteins termed Mas20p, Mas22p, Mas70p and Mas37p. Homologues of the first three proteins have also been found in *Neurospora crassa*, and termed MOM19, MOM22 and MOM72 (reviewed in refs 3, 4). Mas20p has an acidic cytosolic domain and seems to bind the basic mitochondrial targeting signal^{5,6}; Mas22p has acidic domains on both sides of the outer membrane, binds precursors on the outer face of mitochondria⁷ and has been proposed to mediate transfer of the targeting signal across the outer membrane⁸; and Mas37p and Mas70p interact selectively with a subgroup of precursors whose import requires ATP outside the mitochondria^{9–11}.

When purified preadrenodoxin (pAd) was bound to purified MSF, the complex exhibited ATPase activity (Fig. 1a, –) which was completely inhibited by yeast mitochondrial outer-membrane vesicles (Fig. 1a, +OM). Thus the yeast vesicles interact with the pAd-MSF complex similarly to the corresponding vesicles from mammalian mitochondria². Inhibition of the MSF-ATPase was almost completely prevented by pretreating the outer membranes with IgGs against either Mas70p (Fig. 1a, +OM, α70) or Mas37p (Fig. 1a, +OM, α37). IgGs against Mas20p and Mas22p (Fig. 1a, +OM, α20/22) had no effect. Interaction of the MSF-pAd complex with the mitochondrial outer membrane is thus apparently mediated by Mas37p and Mas70p.

Mas37p and Mas70p can be isolated from solubilized outer membranes as a heterodimer¹¹. A detergent extract of outer membranes still blocked the ATPase activity of MSF (Fig. 1b, +OM), and this inhibitory effect was abolished by immunodepleting the Mas37p/Mas70p heterodimer from the extract (Fig. 1b, +OM, 37/70). Immunodepleting Mas20p and Mas22p

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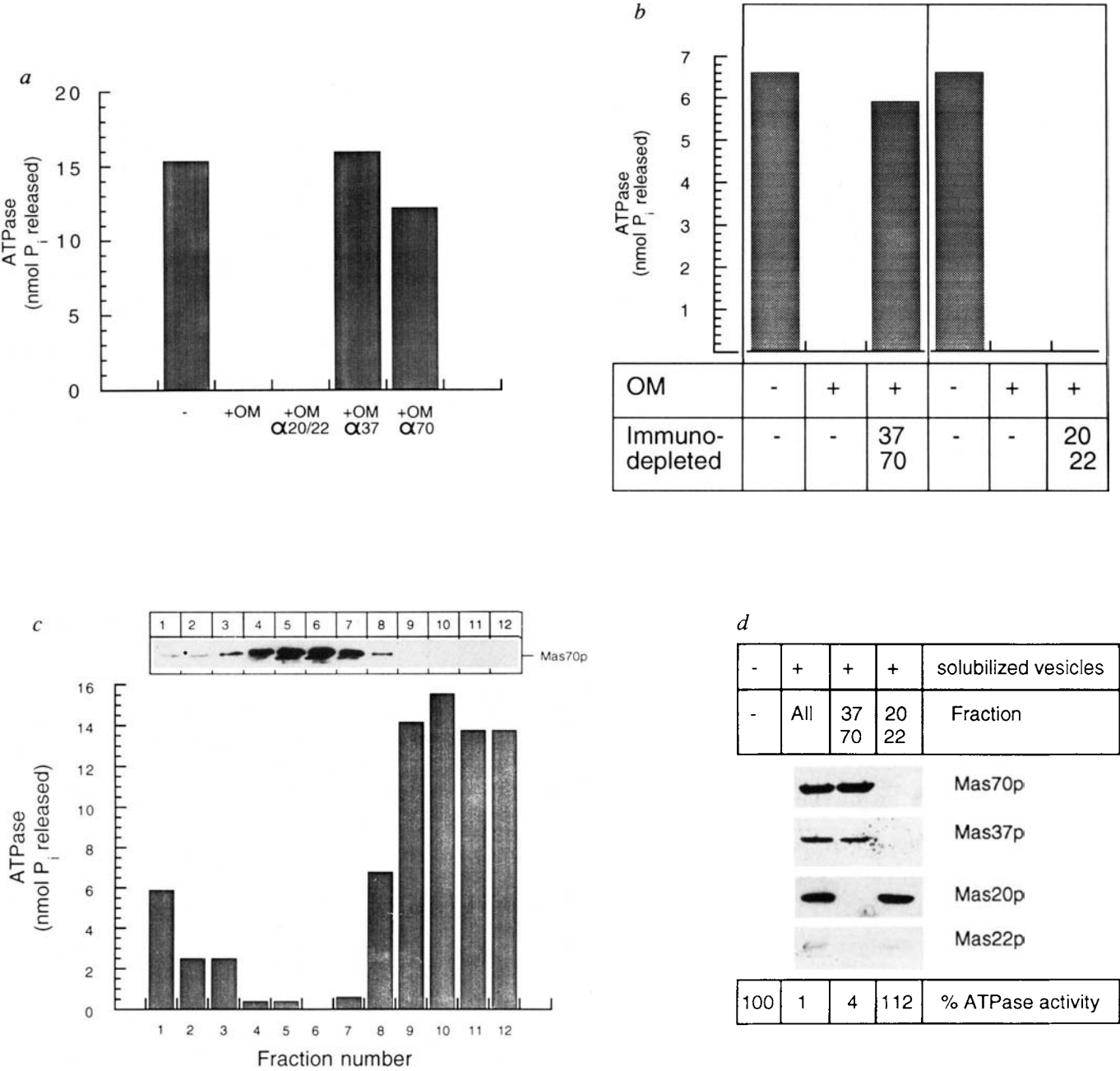


FIG. 1 The Mas70/Mas37 heterodimer in the yeast mitochondrial outer membrane inhibits the ATPase activity of the MSF-pAd complex. **METHODS.** *a*, Preadrenodoxin (1 μ g) and MSF (1 μ g) were incubated for 15 min at 30 $^{\circ}$ C (ref. 1). Yeast mitochondrial outer membranes (0.3 μ g; ref. 12) or outer membranes (0.3 μ g) pretreated for 30 min at 0 $^{\circ}$ C with 20 μ g IgG specific for Mas37p (ref. 11), for Mas70p (ref. 13), or for Mas20p and Mas22p (refs. 8, 14) were then added and the ATPase activity of MSF was determined for 15 min. Purification of MSF and pAd, and the ATPase assay, were as described¹. *b*, Outer membranes were solubilized in 1% (v/v) octylpolyoxyethylene (OPOE), 20 mM HEPES (pH 7.4), 10% (v/v) glycerol, 250 mM NaCl and the protease inhibitors described elsewhere⁹. For immunodepleting Mas20p/Mas22p, solubilization was in 2% OPOE, 50 mM HEPES and 20 mM NaCl. In both cases portions of the extract were incubated with pre-immune IgG(-), IgG against Mas70p (70/37), or IgG against Mas20p and Mas22p (20/22). After incubation with protein-A-Sepharose beads and centrifugation, the supernatants were assayed as in *a*. Immunodepletion was verified by immunoblotting (not shown). Published methods were used for obtaining anti-Mas70p IgGs and IgGs against Mas20p and Mas22p (ref. 14), for immunoblotting¹⁵, and for SDS-PAGE¹⁶. *c*, An extract of outer

membranes in 1% OPOE, 20 mM HEPES-KOH (pH 7.2), 10% glycerol and 250 mM NaCl was centrifuged for 16 h at 259,000g in a 5–20% linear sucrose gradient in solubilization buffer. Fractions were assayed either for inhibition of the ATPase activity of the MSF-pAd complex (lower panel), or for Mas70p by SDS-PAGE and immunoblotting (upper panel). Migration of Mas70p in this system exactly matches that of Mas37p (ref. 11). *d*, An extract of outer membranes in 2% OPOE, 50 mM HEPES-KOH (pH 7.4), 250 mM NaCl, 10% glycerol and 0.5 mM phenylmethyl sulphonyl fluoride (PMSF) was diluted 5-fold with the same buffer lacking NaCl and then chromatographed on a Mono Q anion exchange column, with a linear NaCl gradient (50–1,000 mM) in dilution buffer. Portions of the solubilized outer membranes (All), and of column fractions known from pilot experiments to contain either the peak of Mas20p and Mas22p but no Mas37p and Mas70p (20, 22), or the peak of Mas37p and Mas70p but no Mas20p or Mas22p (37, 70), were tested for inhibition of the ATPase activity of the MSF-pAd complex (lower panel) as outlined in *a* and *b*. Receptor subunits (shown on the right-hand margin) in each fraction were measured by SDS-PAGE and immunoblotting (upper panel).

from the extract did not relieve the inhibition (Fig. 1b, +OM, 20/22).

When the Mas37p/Mas70p heterodimer was partly purified on a detergent-containing sucrose density gradient¹¹, the inhibitory activity copurified with the Mas37p/Mas70p heterodimer (Fig. 1c).

When a detergent extract of outer membranes was fractionated by anion-exchange chromatography, fractions containing Mas20p and Mas22p did not inhibit the ATPase of the pAd-MSF complex (Fig. 1d), whereas fractions containing the Mas37p/Mas70p heterodimer inhibited the ATPase completely (Fig. 1d).

Thus the MSF-pAd complex seems to dock onto the Mas37p/Mas70p subunits of the import receptor, and this docking event quenches the ATPase activity of MSF. To provide direct evidence for such a physical interaction, we bound the MSF-pAd complex to outer membranes, solubilized the mixture under conditions that disrupt interaction between the Mas37p/Mas70p heterodimer and the Mas20p/Mas22p subunits, and checked whether MSF and pAd could be co-immunoprecipitated with IgGs against Mas70p (Fig. 2a). This proved to be so: the immunoprecipitate contained not only Mas70p and its partner subunit Mas37p, but also MSF and pAd (Fig. 2a, lane 3). None of these proteins was precipitated by preimmune-IgGs (Fig. 2a, lane 5). Upon addition of ATP to the extracts, anti-Mas70p IgGs co-precipitated only Mas37p and pAd (Fig. 2b, lane 3) and the MSF heterodimer was released from pAd and the receptor subunits (Fig. 2b, lane 4). None of these proteins was co-immunoprecipitated by anti-Mas20p IgGs (Fig. 2b, lane 5).

To determine the role of Mas37p and Mas70p in the import of pAd in the absence of MSF, radiolabelled urea-denatured pAd was directly diluted into a suspension of energized mitochondria: a significant fraction of the precursor was imported and processed to the mature form (mAd; Fig. 3a, PI). Import was essentially unaffected by IgGs against Mas37p and Mas70p, but was completely blocked by IgGs against Mas20p and Mas22p (Fig. 3a). However, when the urea-denatured precursor was prebound to MSF and then added to mitochondria, its import was completely inhibited by IgGs against Mas37p and Mas70p, and partly inhibited by IgGs against Mas20p and Mas22p (Fig. 3b). The 'naked' precursor thus seems to bind mainly to the acidic subunits (Mas20p and probably also

Mas22p) of the import receptor. In contrast, the pAd-MSF complex binds to Mas37p/Mas70p; MSF is then released and the precursor transferred across the outer membrane with the assistance of Mas20p and Mas22p.

Sequential transfer of pAd from MSF to the various receptor subunits is also indicated by the chase experiment shown in Fig. 3c, d. Radiolabelled pAd, either free or complexed to MSF, was bound to mitochondria that had been de-energized and pre-treated with pre-immune IgGs, IgGs against Mas37p/Mas70p, or IgGs against Mas20p/Mas22p. A portion of each mitochondrial sample was then analysed for bound precursor (Bind). Another portion was re-energized, incubated for 4 min at 30 °C to chase prebound precursor into the mitochondria, and then analysed for imported precursor (Chase). With the 'naked' precursor, anti-Mas20p/Mas22p IgGs strongly inhibited binding of the precursor, none of the residual amount of bound precursor could be chased, and neither binding nor chase was affected by anti-Mas37p/Mas70p IgGs (Fig. 3c). With the MSF-bound precursor, binding was strongly inhibited by anti-Mas37p/Mas70p IgGs, but not by anti-Mas20p/Mas22p IgGs. However, chasing of prebound precursor was inhibited by anti-Mas37p/Mas70p/IgGs as well as by anti-Mas20p/Mas22p IgGs (Fig. 3d).

These results suggest the model shown in Fig. 4. Many newly made precursor proteins are bound by cytosolic chaperones¹². Some of these chaperones, such as MSF, selectively bind precursors with mitochondrial targeting sequences. By hydrolysing ATP, the chaperone keeps the precursor competent for docking to the import receptor subunits Mas37p and Mas70p. MSF alone does not bind to mitochondrial outer membranes from rat liver² or yeast (N.H., unpublished observations). However, the MSF-precursor complex binds to Mas37p/Mas70p in the absence of ATP, forming a stable MSF-precursor-Mas37p/Mas70p complex. In the presence of ATP, MSF dissociates from these receptor subunits and the precursor's presequence is transferred from Mas37p/Mas70p to Mas20p and Mas22p and then across the outer membrane to the acidic Mas22p domain in the intermembrane space. The reconstituted assay systems described here measure the first two steps of this pathway. The highly active ATPase activity of the MSF-pAd complex presumably reflects multiple rounds of ATP-dependent release of precursor from MSF followed by rebinding. When the precursor is trans-

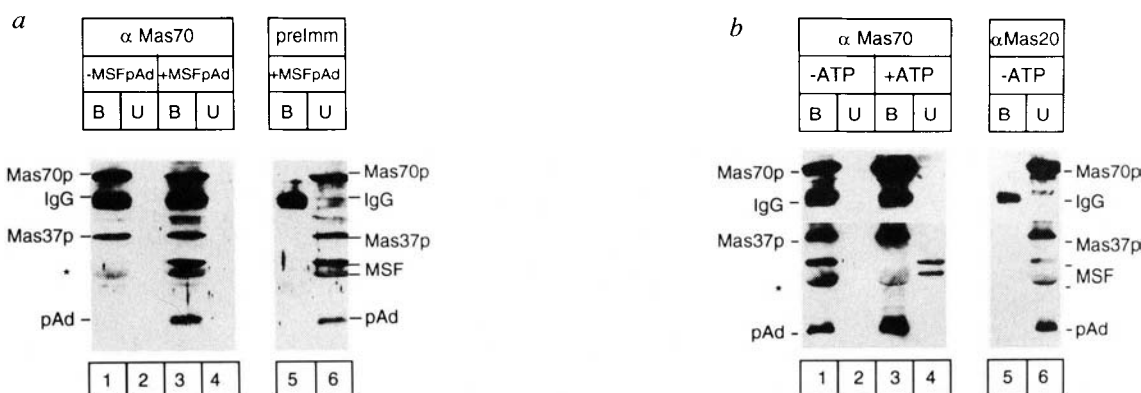


FIG. 2 MSF and pAd form an ATP-sensitive complex with the Mas37p/Mas70p heterodimer.

METHODS. Outer membranes were solubilized with OPOE as in Fig. 1c. One aliquot of the extract (10 µg) was incubated for 30 min at 30 °C with MSF-pAd complex (1 µg of each component) (+MSFpAd) in the absence (a, lanes 3-6; b, lanes 1, 2, 5, 6) or presence (b, lanes 3, 4) of 1 mM ATP; the other aliquot was left untreated (a, lanes 1, 2). All aliquots were incubated overnight with protein-A-Sepharose beads

containing immobilized preimmune IgG (prelmm), IgG against Mas70p, or IgG against Mas20p plus Mas22p (see top of each panel). Bound (B) and unbound (U) material was analysed by SDS-PAGE and immunoblotting with the antisera indicated on the margin. The band marked IgG represents the heavy IgG chain. MSF consists of two closely spaced subunits of relative molecular mass (M_r) 30K and 32K (ref. 2). Asterisked is an unspecific reaction product of one of the antisera migrating slightly, but distinctly, faster than the smaller MSF subunit.

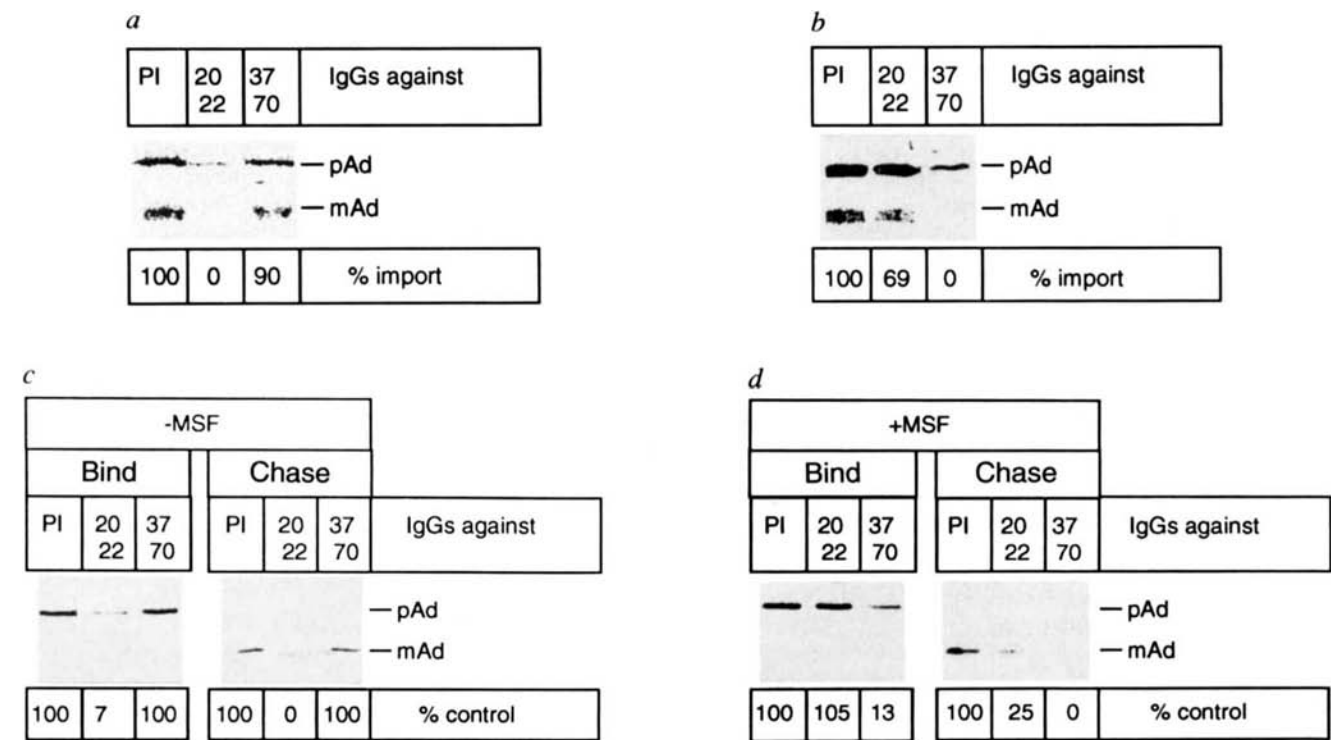


FIG. 3 Sequential binding of MSF-bound precursor to Mas37p/Mas70p and Mas20p/Mas22p. **METHODS.** Preadrenodoxin was synthesized in a wheat-germ lysate in the presence of [³⁵S]methionine¹. The lysate was centrifuged, and the precursor was precipitated from the supernatant with 2 volumes of saturated ammonium sulphate, dissolved in 8 M urea, 20 mM HEPES–KOH (pH 7.6) and incubated for 10 min at 0 °C. **a**, The urea-denatured precursor was diluted 40-fold into 100 µl import buffer containing 1.0 µg of purified mitochondria¹⁶ that had been pretreated for 30 min at 0 °C with 100 µg of the following IgGs: preimmune IgG (PI), IgG against Mas37p and Mas70p (37/70), or IgG against Mas20p and Mas22p (20/22). After 4 min at 30 °C, the mitochondria were reisolated, and import of pAd was assayed by SDS–PAGE and scanning of the dried gel in a phosphorimager. Import in the presence of preimmune serum was taken as 100%. **b**, As in **a**, but the urea-denatured precursor was first

diluted 100-fold into a solution containing MSF (3.5 µg ml⁻¹) in import buffer, followed by the addition of mitochondria. **c**, Mitochondria were first incubated with preimmune IgGs, anti-Mas37p/Mas70p IgGs, or anti-Mas20p/Ms22p IgGs, then uncoupled with 5 µM carbonyl cyanide *m*-chlorophenylhydrazide, and finally mixed with urea-denatured wheatgerm lysate containing radiolabelled pAd (–MSF). After 4 min at 30 °C, a portion was centrifuged to analyse binding of precursor to the mitochondria. The other portion of mitochondria was re-energized¹⁷, chased for 4 min at 30 °C, treated with proteinase K (100 µg ml⁻¹) followed by PMSF, and reisolated by centrifugation. Mitochondria from both portions were then subjected to SDS–PAGE and analysis of the dried gels in a phosphorimager. **d**, As in **c**, except that the same amount of urea-denatured radiolabelled precursor was first bound to MSF before being mixed with the mitochondria (+MSF).

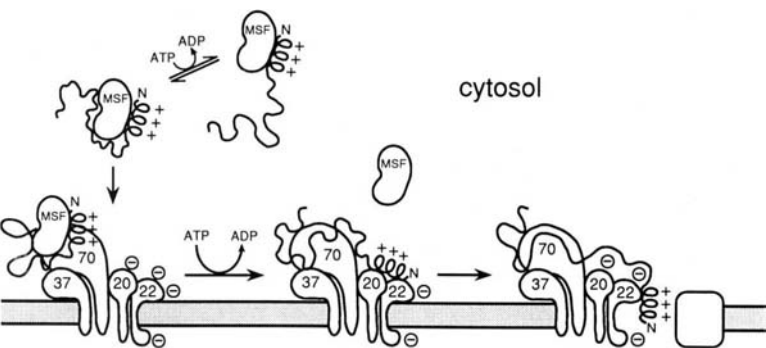


FIG. 4 The early steps of mitochondrial protein import. The model implies that Mas22p associates at least transiently with the import channel in the outer membrane.

ferred to Mas37p/Mas70p, the ATPase of MSF is blocked, because the single round of ATP hydrolysis required to transfer the precursor from MSF to Mas37p/Mas70p is below the detection limit of our assay system. MSF is only one of several cytosolic chaperones that mediate the import of proteins into mitochondria. It will be interesting

to learn how other molecular chaperones assist in the import of mitochondrial precursor proteins. □

Received 19 June; accepted 19 July 1995.
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ACKNOWLEDGEMENTS. We thank W. Oppliger for technical assistance; M. Jäggi, L. Müller and V. Grieder for the artwork; and S. Gratzner and S. Kohlwein for antibodies. G.S. was supported by the Swiss National Science Foundation and the HCM program of the EEC, and T.L. by a fellowship from the Human Frontiers Science Program Organization.

A cap-binding protein complex mediating U snRNA export

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CAP structures are added cotranscriptionally to all RNA polymerase II transcripts^{1,2}. They affect several processes including RNA stability^{3–5}, pre-messenger RNA splicing^{6–11}, RNA export from the nucleus^{12–14} and translation initiation^{15–17}. The effect of the cap on translation is mediated by the initiation factor eIF-4F (refs 15–19), whereas the effect on pre-mRNA splicing involves a nuclear complex (CBC) composed of two cap binding proteins, CBP80 and CBP20¹¹. A role for CBC in the nuclear export of capped RNAs has also been proposed^{11,13}. We report here the characterization of human and *Xenopus* CBP20s. Antibodies against recombinant CBP20 prevent interaction of CBC with capped RNAs *in vitro*. Following microinjection into *Xenopus* oocytes, the antibodies inhibit both pre-mRNA splicing and export of U small nuclear RNAs to the cytoplasm. These results demonstrate that CBC mediates the effect of the cap structure in U snRNA export, and provide direct evidence for the involvement of a cellular RNA-binding factor in the transport of RNA to the cytoplasm.

Human CBC consists of a heterodimeric complex of CBP80 and CBP20¹¹. The cloning and characterization of CBP80 has been reported previously^{11,20}. CBP20 was purified and partially sequenced, and the sequence used to design oligonucleotide primers that enabled the isolation of complementary DNAs from a HeLa cell cDNA library. The longest open reading frame (ORF) in the cDNAs encodes a 156 amino-acid protein with a calculated relative molecular mass (M_r) of 17.98K. Translation of the ORF *in vitro* generates a protein that comigrates with purified CBP20 on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; data not shown), and interacts specifically with CBP80 (see below). Antibodies raised against the recombinant protein recognized purified HeLa cell CBP20 on western blots (data not shown). This evidence shows that the cDNAs encode CBP20. The predicted amino-acid sequences of human CBP20 and its *Xenopus* homologue are shown in Fig. 1. The protein is highly conserved between the two species. The *Xenopus* cDNA is incomplete at its amino terminus but, in the

147 amino-acid positions that can be compared, *Xenopus* and human CBP20 show 84% identity and 99% similarity. CBP20 belongs to the large family of proteins containing a conserved (RNP) motif of roughly 80 amino acids including the highly conserved RNP1 and RNP2 sequences^{21,22}. In spite of this, recombinant CBP20 did not exhibit detectable nonspecific RNA binding activity or specific cap binding activity in the absence of CBP80 (data not shown). However, when recombinant CBP20 was copurified with CBP80 (note that this purification relies on specific interactions between the two proteins; see Fig. 2) cap-specific binding similar to that attributable to the CBC present in HeLa cell nuclear extracts^{11,13,23} was observed (Fig. 2, lanes 2 and 8).

As expected from the conservation of CBP20, polyclonal antibodies raised against human CBP20 produced in *Escherichia coli* recognized both human and *Xenopus* CBP20 obtained by *in vitro* translation. When used in western blots the antibodies labelled a major band with the expected mobility in HeLa cell and *Xenopus* oocyte lysates (data not shown). After affinity purification, these antibodies prevented CBC binding to a capped RNA probe *in vitro* (Fig. 2, lanes 3 and 9) and the inhibitory effect could be relieved by the addition of excess recombinant CBP20 to the reaction (Fig. 2, lanes 4 and 10). The lower mobility cap-specific complex seen in HeLa cell nuclear extracts (Fig. 2, lanes 2, 4 and 5; and ref. 13) is also abrogated by the antibodies, indicating that it also contains CBP20, but its composition has not been further analysed.

To test if these antibodies could also prevent CBC interaction with capped RNAs *in vivo* its effect on splicing was investigated. The role of CBC in pre-mRNA splicing was demonstrated *in vitro* by immunodepletion of HeLa nuclear extracts using antibodies raised against CBP80 (ref. 11). Affinity-purified anti-CBP20 antibodies inhibited splicing both *in vitro* when added

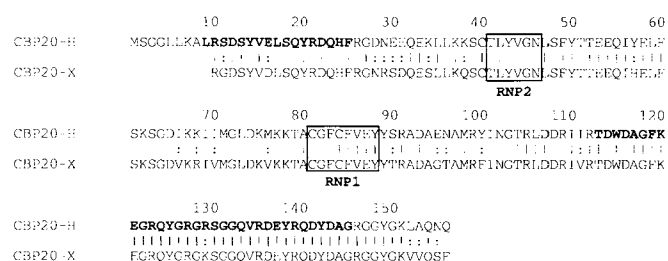


FIG. 1 Deduced amino-acid sequence of human CBP20 aligned with its *Xenopus laevis* homologue. The predicted amino-acid sequence of human CBP20 (top, CBP20-H) was aligned with the partial *Xenopus* protein sequence (bottom, CBP20-X) using the gap-program²⁸. CBP20-H amino acids are numbered. Identical and similar residues between the two proteins are indicated by lines and dots, respectively. Sequences obtained from tryptic peptides of purified human CBP20 are indicated in bold. The conserved RNP1 and RNP2 motifs^{21,22} are boxed. METHODS. Human CBP20 was purified from HeLa nuclear extracts and microsequenced as described for CBP80¹¹. A partial cDNA clone containing the C-terminal end of the coding region and 150 bp of the 3'-untranslated region were obtained by 3'-RACE²⁹ using a degenerate oligonucleotide encoding the peptide encompassing amino acids 133 to 138. The products of the reaction were cloned into pBluescribe, sequenced and selected on the basis of the match of the amino-acid codons following residue 138 to the peptide sequences. One of the partial cDNAs was then used as probe to screen a randomly primed HeLa cell cDNA library. One of the isolated cDNAs contains an open reading frame encoding the complete protein sequence. The *in vitro* translation product programmed by this cDNA showed the same mobility as purified human CBP20 on SDS-PAGE, and polyclonal antibodies raised against the recombinant protein produced in *E. coli* recognized both the *in vitro* translated protein and the purified human protein (data not shown) confirming that the isolated cDNA encodes authentic CBP20. This clone was then used as probe to screen a *Xenopus laevis* cDNA library. Library screening and purification of positive bacteriophage plaques were performed according to standard protocols.

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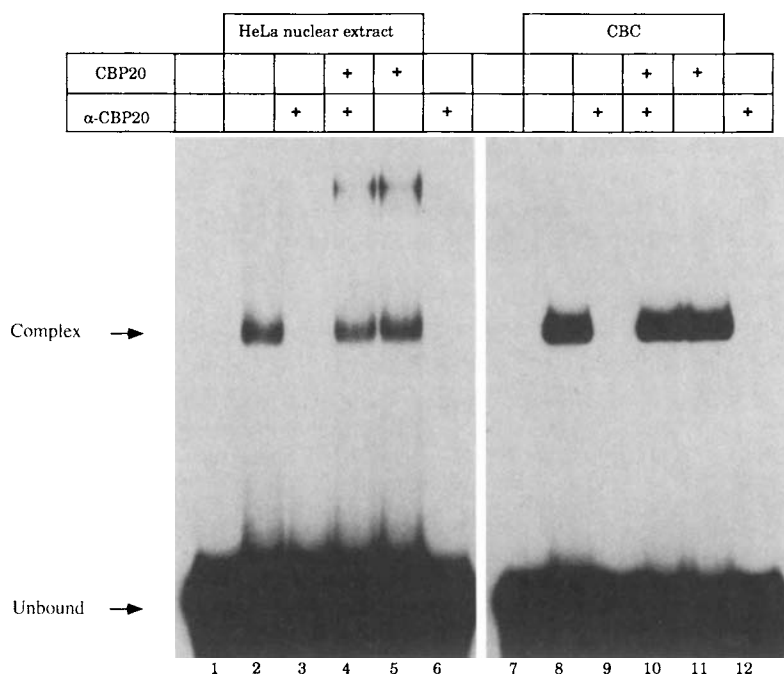
† To whom correspondence should be addressed.

directly to HeLa nuclear extracts (data not shown), and *in vivo*, after microinjection into *Xenopus* oocytes (Fig. 3, lanes 1–4). As compared to control oocytes or oocytes injected with pre-immune antibodies, those injected with anti-CBP20 antibodies exhibited significantly reduced splicing activity, although in most experiments splicing activity was not completely abolished (data not shown). The specificity of the effect was demonstrated by reversing the inhibition by pre-incubation of the injected antibodies with an excess of recombinant CBP20 (Fig. 3, lane 5). CBP20 itself had no effect on splicing (lane 6). These data provide evidence for the role of CBC in splicing *in vivo* and suggest that the CBP20 antibodies reduce CBC binding to pre-mRNA in *Xenopus* oocytes. Because previous data indicated that the

cap binding activity involved in U snRNA export was similar to that which functions in splicing^{11,13}, we next tested the effect of anti-CBP20 antibodies on U snRNA export. Following pre-injection of affinity-purified CBP20 antibodies, a mixture of four RNAs were microinjected into *Xenopus* oocyte nuclei. These were: mutants of U1 and U5 snRNAs that are exported normally but, unlike wild-type U1 and U5, are not subsequently reimported to the nucleus^{12,14}; U6 Δ ss RNA, an RNA that is not significantly exported from the nucleus and serves as a control for accurate nuclear injection (24); and human initiator methionyl transfer RNA, an RNA that is exported from the nucleus in a cap-independent manner^{14,25}. Immediately after injection, all four RNAs were nuclear (Fig. 4a, lanes 1–3). After

FIG. 2 Anti-CBP20 antibodies prevent CBC binding to a capped RNA *in vitro*. Rabbit antibodies were raised against a histidine-tagged CBP20 protein expressed in *E. coli*. An electrophoretic mobility retardation assay was performed as described¹³ with either HeLa nuclear extract (lanes 1–6) or recombinant CBC (lanes 7–12). Lanes 1 and 7: free RNA. Lanes 2 and 8: CBC–RNA complexes formed when the capped RNA probe was incubated with HeLa nuclear extract or recombinant CBC, respectively. The position of the free RNA probe and of the CBC–RNA complex is indicated on the left. The upper complex seen in lanes 4 and 5 was previously observed in similar experiments¹³, and is thought to represent a higher-order CBC complex. Lanes 3 and 9 show that when affinity-purified CBP20 antiserum was included in the reaction, formation of CBC–RNA complexes was prevented. This inhibitory effect was abolished if a CBP20–GST fusion protein was added together with the serum to the binding reaction (lanes 4 and 10). Controls show that the fusion protein has no effect on complex formation and that the antibody on its own has no effect on RNA mobility (lanes 5, 6, 11, 12). Lanes 2 to 5 contain 0.8 μ g total nuclear protein. Lanes 8 to 11 contain approximately 50 ng of recombinant complex. Lanes 3, 4, 6, 9, 10 and 12 contain 1 μ g affinity-purified anti-CBP20 antibodies. Lanes 4, 5, 10 and 11 contain 2 μ g CBP20–GST fusion proteins.

METHODS. Human CBP20 was expressed in *E. coli* either as a histidine-tagged protein using the pRSETA vector system (Invitrogen Corporation) or as a glutathione-S-transferase fusion protein using the pGEX-2T vector (Pharmacia). Human CBP80 was expressed using the pT7-7TT vector³⁰. Histidine-tagged CBP20 was purified on a nickel-NTA-agarose column (Qiagen). To prepare recombinant CBC, lysates from *E. coli* expressing human CBP80 were obtained by sonication of bacterial pellets in PGK buffer (50 mM sodium phosphate, pH 7.2, 40% glycerol, 0.1 M KCl, 0.5% Triton X-100, 25 mM imidazole). Lysates were cleared by centrifugation at 15,000 r.p.m. for 20 min in a SS-34 rotor. Purified histidine-tagged CBP20 together with nickel-NTA-agarose beads were then added and



the mixture was rotated for 1 h at room temperature. Beads were washed three times with PGK buffer and poured into a column. Elution of recombinant complex was performed with PGK buffer containing 0.5 M imidazole. Anti-CBP20 antisera were raised against histidine-tagged CBP20 expressed in *E. coli*. The antibodies were purified over an affinity column made by coupling the GST–CBP20 fusion protein to Affigel-10 beads (BioRad).

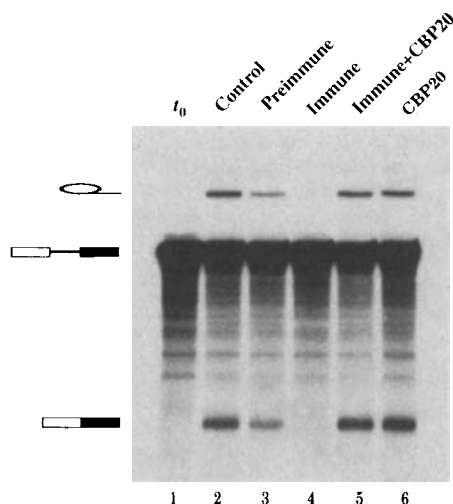


FIG. 3 Anti-CBP20 antibodies inhibit splicing *in vivo* in *Xenopus* oocytes. *Xenopus laevis* oocyte nuclei were injected with affinity-purified anti-CBP20 antibodies (lane 4) or with a mixture of antibodies and recombinant CBP20–GST fusion protein (lane 5). As controls, oocytes were injected either with PBS (lanes 1 and 2), preimmune serum purified following the same procedure as for the immune serum (lane 3), or with recombinant CBP20–GST protein (lane 6). After 1 h incubation, a second microinjection was performed with radioactively labelled pBSAd1 precursor RNA¹¹ synthesized by T3 polymerase primed with the m⁷GpppG cap dinucleotide. In lanes 2 to 6 RNA was extracted 60 min after injection; in lane 1, RNA was extracted immediately after injection. The precursor and products of the splicing reaction are indicated diagrammatically on the left.

METHODS. Affinity purification of preimmune and immune sera and preparation of recombinant CBP20–GST fusion protein were carried out as described in the legend to Fig. 2. The immunoglobulin concentration in the injected samples was 4 μ g μ l⁻¹ and that of the CBP20–GST recombinant protein 2 μ g μ l⁻¹.

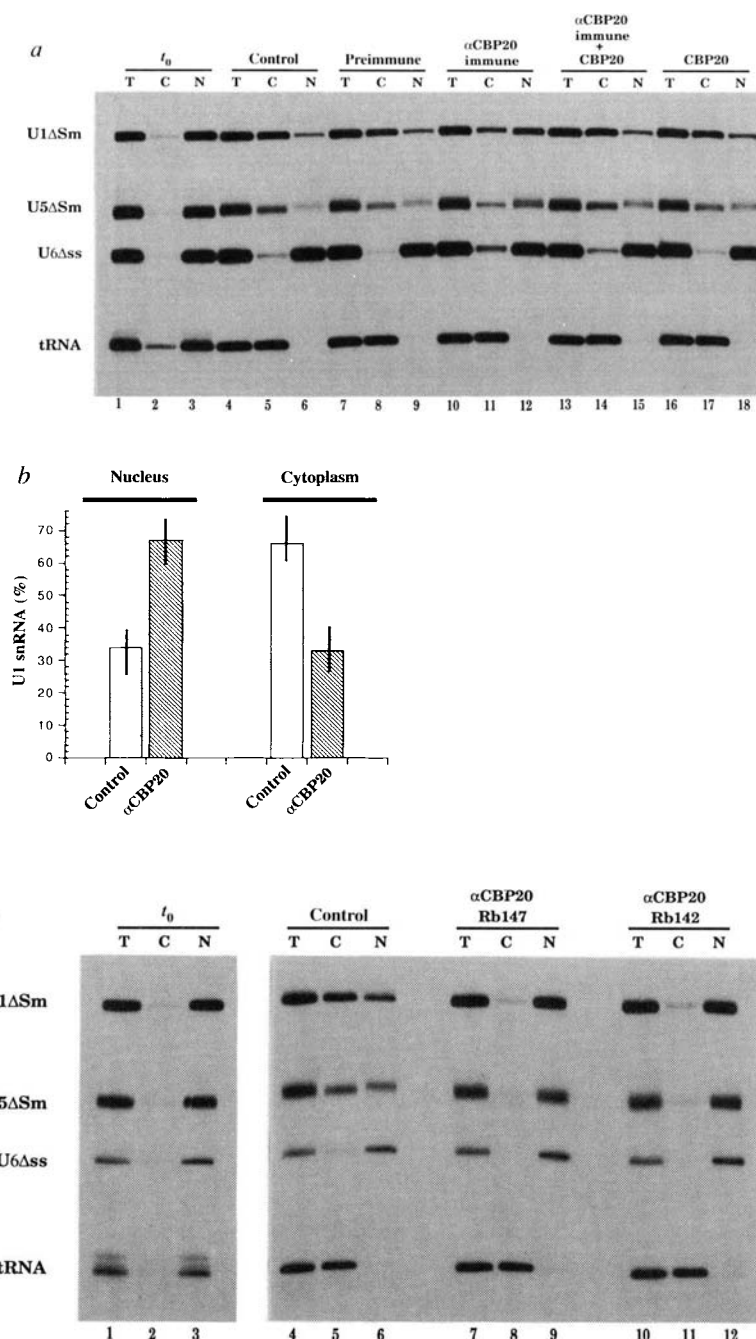
160 min incubation U1, U5 and tRNA were largely cytoplasmic, whereas U6 was retained in the nucleus (lanes 4–6). Injection of either pre-immune antibodies or of recombinant CBP20 had no significant effect on export (lanes 7–9, 16–18). Microinjection of CBP20 antibodies, however, resulted in significant inhibition of U1 and U5 snRNA export (Fig. 4a, compare lanes 1–9 with 10–12), whereas export of tRNA and retention of U6 RNA were not affected. The inhibition of U snRNA export was specific, because pre-incubation of the antibodies with recombinant CBP20 reversed the effect (lanes 13–15).

The inhibition of U1 and U5 export was not complete, but was very reproducible using several different preparations of affinity-purified antibodies. The effect of anti-CBP20 antibodies on the export of U1 in five independent experiments was quantified and is graphically represented in Fig. 4b. The mean values for the nuclear and cytoplasmic fraction of the RNA in the absence and presence of anti-CBP20 are shown, with the error

bars representing the maximal and minimal values obtained in the five experiments. Although the antibodies have a considerable effect on U1 snRNA export, the inhibition is not complete. It may be that the anti-CBP20 antibodies do not completely saturate the CBC *in vivo* because of an insufficient titre or affinity, or because of the interaction of CBC with additional factors that partially block access of the antibody to the complex. Alternatively, CBC may not be absolutely required for U snRNA export. We note, however, that the export of U snRNAs can be completely inhibited by short capped competitor RNAs¹⁴ which suggests that factors that recognize the cap structure are indeed essential.

Although the effects of the anti-CBP20 antibodies appeared specific (Fig. 4a) we wished to eliminate the possibility that the inhibitory effects observed might be due to some unexpected property of the antiserum used. To this end a distinct anti-CBP20 antiserum was raised. Affinity-purified antibodies from

FIG. 4 Anti-CBP20 antibodies inhibit U snRNA export *in vivo* in *Xenopus* oocytes. **a**, *Xenopus laevis* oocyte nuclei were injected with affinity-purified anti-CBP20 antibodies (lanes 10 to 12) or with a mixture of antibodies and recombinant CBP20–GST fusion protein (lanes 13 to 15). As controls oocytes were injected either with PBS (lanes 1 to 6), preimmune serum purified following the same procedure as for the immune serum (lanes 7 to 9), or with recombinant CBP20–GST protein (lanes 16 to 18). After 1 h incubation, a second microinjection was performed with a mixture of the following radioactively labelled RNAs: U1ΔSm¹², U5ΔSm¹⁴, U6Δss²⁴ and human initiator methionyl tRNA²⁵. Synthesis of U1ΔSm and U5ΔSm RNAs was primed with the m⁷GpppG cap dinucleotide, whereas synthesis of U6Δss RNA was primed with γmGTP. In lanes 4 to 18 RNA was extracted 160 min after injection; in lanes 1 to 3, RNA was extracted immediately after injection. **b**, The percentage of U1ΔSm snRNA in the nuclear and cytoplasmic fractions of oocytes was determined 150 min after its injection into the nucleus. Either control oocytes (white bars) or oocytes that had previously been microinjected with affinity-purified anti-CBP20 antibodies (cross-hatched bars) were analysed. The error bars represent the maximum and minimum values obtained in five separate experiments. **c**, The export of U1ΔSm, U5ΔSm and tRNA was analysed 120 min after their microinjection into the nuclei of oocytes as in **a**. U6Δss is an internal control for nuclear integrity. Oocyte nuclei were preinjected 1 h before the RNA injection with either PBS (lanes 4–6) or with affinity-purified anti-CBP20 antibodies from rabbit 147 (as in Figs 2–4b) or rabbit 142. **METHODS.** Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis, autoradiography or phosphorimager analysis were performed as described in ref. 14. The immunoglobulin and recombinant protein concentrations in the injected samples were the same as described in the legend to Fig. 3.



this second serum prevented CBC interaction with capped RNA *in vitro* (data not shown) and, when microinjected into oocyte nuclei, inhibited U1 and U5 snRNA export similarly to antibodies from the first serum (Fig. 4c, compare the control lanes 4–6 with lanes 7–9 and 10–12). This strongly supports the proposed role of the CBC in U snRNA export. In similar experiments, U2 and U4 snRNA export was also found to be inhibited, but we have thus far failed to see an effect of the antibodies on mRNA export (data not shown). However, because the effect of the cap structure on mRNA export is much less dramatic than in the case of U snRNAs¹⁴, much more careful kinetic analysis, and perhaps also antibody preparations that produce

stronger inhibitory effects on U snRNA export, will be required to evaluate the possible role of CBC in mRNA export.

In conclusion, these experiments provide strong evidence that CBP20, as part of the nuclear CBC, is directly involved in mediating U snRNA export from the nucleus. There are currently no other cellular RNA-binding proteins for which evidence of this nature is available, but HIV Rev protein was recently shown to have a direct role in stimulating RNA export from the nucleus²⁶. Rev is an RNA-binding protein encoded by the HIV virus²⁷, and it is possible that the mechanism of action of CBC and Rev will exhibit similarities. □

Received 17 March; accepted 16 June 1995.

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ACKNOWLEDGEMENTS. E.I. and J.L. contributed equally to this work. We thank W. Boelens and I. Palacios for help with oocyte injections; A. Miller for HeLa cells; S. Gunderson, A. Lamond, M. Polycarpou-Schwarz, B. Séraphin and K. Weis for comments on the manuscript; P. Chambon and R. Stick for HeLa and *Xenopus laevis* cDNA libraries, respectively; the EMBL and Harvard protein sequencing services for CBP20 peptide sequences; M. Jankowska and E. Darzynkiewicz for cap analogues; and EMBL services for help in preparing the manuscript. The accession number for the sequences reported in this paper are X84157 and X84788, for human and *Xenopus* CBP20, respectively.

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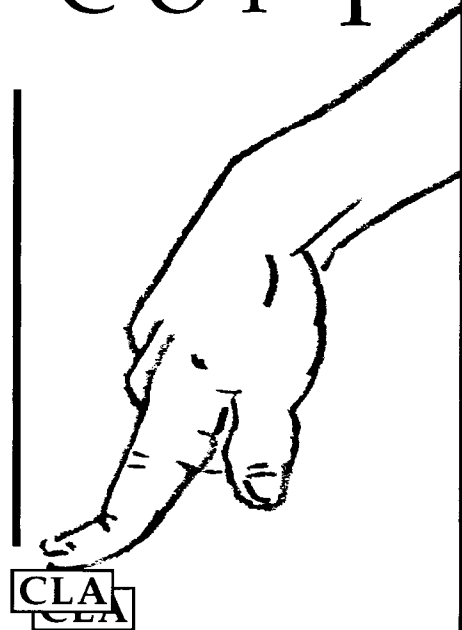
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